IN VIVO AND *IN VITRO* EVIDENCE FOR THE SYNTHESIS AND PROCESSING OF A STYRENE OXIDE INDUCED FORM OF EPOXIDE HYDROLASE

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ABSTRACT

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IN VIVO AND IN VITRO EVIDENCE FOR THE SYNTHESIS AND PROCESSING Of A STYRENE OXIDE INDUCED FORM OF EPOXIDE HYDROLASE

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In vivo and *in vitro* studies have been done to determine the site of biosynthesis and processing of styrene oxide (SO) induced epoxide hydrolase (EH). Adult male Long-Evans rats were used to induce a form of epoxide hydrolase using styrene oxide as the inducing agent. Rats were administered 500 milligrams of SO per milliliter of corn oil intraperitoneally per day. They were sacrificed forty-eight hours post-injections and the livers were quickly removed, homogenized and fractionated on a sucrose discontinuous density gradient to obtain the microsomal fraction. Epoxide Hydrolase was synthesized in a cell-free system using a rabbit reticulocyte lysate system. EH_{SO} was partially purified using lubrol - WX detergent solubilization and ion exchange chromatography. Analysis of EH_{SO} using SDS-PAGE showed the *in vivo* synthesis of a microsomal form of the enzyme. Autoradiography of the *in vitro* synthesis product (EH_{SO}) showed that the enzyme is processed on RER-membrane bound polysomes. The results suggest that EH_{SO} is synthesized on membrane-bound polysomes and follows the regulatory processing pathway of secretory proteins and, putatively, integral membrane proteins.

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LIST OF ABBREVIATIONS

CP----------Creatine phosphate

DPM----------Stripped dog pancreatic microsomal membrane

EDTA----------Ethylenediamine tetracetic acid

EH_{SO}----------Styrene oxide induced epoxide hydrolase

FP----------Free polysomes

MBP----------Membrane-bound polysomes

3-MC----------3-methylcholanthrene

PAGE----------Polyacrylamide gel electrophoresis

PMS----------Post-mitochondrial supernatant

PNS----------Post-nuclear supernatant

PB----------Phenobarbital

RER----------Rough endoplasmic reticulum

RER-MBP----------RER-membrane bound polysomes

RM----------Rough microsomes

SDS----------Sodium dodecyl sulphate

SDS-PAGE----------Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SM----------Smooth microsomes

SO----------Styrene Oxide

TSO----------Trans-stilbene oxide

CHAPTER I

INTRODUCTION

In today's environment there are many pollutants in the soil, waterways and atmosphere which are potential mutagens and/or carcinogens. Many of these compounds exist as aromatic or olefinic compounds which are biotransformed in mammals by enzymes localized in the endoplasmic reticulum. Membrane-bound monooxygenases can catalyze these compounds to epoxides and arene oxides, which in most cases are highly reactive electrophiles and can be hydrated enzymatically via epoxide hydrolase.

Microsomal epoxide hydrolase (E.C.3.3.2.3) is a family of integral membrane enzymes of the endoplasmic reticulum responsible for the metabolism of many endogenous and exogenous lipophilic compounds, some of which may be mutagenic and/or carcinogenic (Conney, 1967, Miller and Miller, 1981, Lorenz et al, 1984). Epoxide hydrolase converts epoxides to dihydrodiols, which is a very important step in the detoxication of these compounds. However, many investigators have also shown that epoxide hydrolase converts many of these compounds (the polycyclic aromatic hydrocarbons are the most extensively studied) to more reactive "bay region" diol epoxides (Pelkonen and Nebert, 1982) which are more toxic than the parent structure. This phenomenom, (duality of function in detoxication and toxication of some compounds) of epoxide hydrolase has created great interest in understanding the role of xenobiotic metabolizing enzymes in the cell.

Presently, a large number of investigators have induced, isolated and purified epoxide hydrolase from liver microsomes of man and a variety of other mammals in order to evaluate its dual role in the cell's defense mechanism. A great deal of information has been obtained concerning the enzyme's role in the metabolism of compounds, however, an important question not answered completely concerns the biosynthesis and mechanism of insertion of this protein into the endoplasmic reticulum. Presumably, during the biosynthesis of this membrane bound protein, cotranslational modifications occur leaving an intact putative noncleavable amino terminal signal sequence that remains in the mature protein during membrane insertion (Okada et al 1982, Ohlsson et al 1981).

Epoxide hydrolase may follow the predictions of the "signal hypothesis," which is the case in many secretory and putatively integral membrane proteins. According to the "signal hypothesis," proteins that are secreted or destined for insertion into the endoplasmic reticulum membrane in eukaryotic cells are synthesized as precursors with an extra NH_2 terminal sequence. This extra sequence consists mainly of 15-20 hydrophobic amino acids (Milstein et al, 1972; and Blobel and Dobbertstein, 1975a). The signal sequence initiates binding of the translational complex to the endoplasmic reticulum, facilitating the passage of the protein through a putative tunnel in the membrane as it is translated. The destiny of the protein determines the fate of the signal sequence

attached. If the protein is to be secreted (secretory and certain integral membrane proteins), the signal sequence is removed from the growing polypeptide during the transfer by a putative signal peptidase, giving rise to the final product (authentic protein). This protein is localized on the luminal surface of the endoplasmic reticulum prior to translocation (Blobel, 1980; Blobel and Dobberstein, 1975b). However, some integral membrane proteins retain their signal sequences (Sabatini et al, 1982). Many investigators have attempted to elucidate the functional role of this sequence in determining the intracellular topography of such proteins (Seidegard et al, 1982).

In our laboratory, we have investigated the in vivo and in vitro aspects of the biogenesis of epoxide hydrolase in rat liver microsomes following exposure to the direct acting carcinogen, styrene oxide. We have assessed the cellular mechanisms of the biosynthesis of epoxide hydrolase following 48 hr exposure to styrene oxide. We have determined if the form of the enzyme identified follows the regulatory processing pathway of previously reported integral membrane proteins (Blobel, 1980). The results obtained provide a better understanding of the dual role of epoxide hydrolase in cellular defense against a potential cancer-causing agent. This, in turn, may provide a better understanding of the cellular and biochemical mechanisms involved in toxicity, mutagenesis, and/or carcinogenesis.

The purpose of this investigation was to determine the site of biosynthesis and mechanism of insertion of one form of epoxide hydrolase into the endomembrane system. Our goal was to provide supportive evidence as to whether or not this enzyme follows the "signal hypothesis" for insertion into the endoplasmic reticulum. Overall, this investigation provides basic information on the induction, biosynthesis and mechanism of insertion of a styrene oxide induced form of epoxide hydrolase and the effects of this carcinogen on the cell's protein synthesizing machinery.

Therefore the objectives of this research are as follows:

1. To isolate and partially purify epoxide hydrolase from styrene oxide treated rat liver.
2. To compare the changes in the protein concentration of the smooth and rough microsomes of the styrene oxide treated liver with untreated liver during the biosynthesis of epoxide hydrolase.
3. To synthesize epoxide hydrolase in a cell-free system and compare it to the authentic molecule to determine its site of biosynthesis.

CHAPTER II

REVIEW OF LITERATURE

The literature discussed here covers the following aspects of the enzyme, microsomal epoxide hydrolase: 1) its functional role in the cell; 2) its purification and properties; 3) its distribution and location; 4) its membrane topology; and 5) its regulation and biosynthesis.

Functional Role in the Cell

Epoxide hydrolase (E.C.3.3.2.3.) is an enzyme found in a variety of mammals, lower animal species (Walker et al, 1978, Walz et al 1983 Mertes et al, 1985) and some plants (Banthorpe and Osborne, 1984). Epoxide hydrolase catalyzes the hydration of a wide range of reactive epoxides to the corresponding trans-dihydrodiols. Epoxides are metabolically formed from many olefinic or aromatic moieties (Daly et al, 1972). Some of these epoxides are reactive electrophiles which can bind covalently to cellular macromolecules and produce toxic, mutagenic and/or carcinogenic effects. The enzymatic hydration of epoxides to less chemically reactive trans-dihydrodiols by the enzyme epoxide hydrolase has been recognized as a major mechanism of detoxication and cellular defense (Jerina et al, 1968; Lu et al 1975 and Oesch et al 1974). However, several investigators (Huberman et al 1971, 1976; Wood et al

1976; Kapitulnik et al 1978; and Geacintov et al, 1984) have shown that certain trans-dihydrodiols derived from some polycyclic aromatic hydrocarbons undergo further metabolism to reactive epoxides by the cytochrome P-450 system. These highly reactive dihydrodiol epoxides may be ultimate carcinogens. Gozurkara et al (1980) showed that these metabolites bind covalently to DNA, in vivo and in vitro, in tissue culture, in isolated rat liver and lung nuclei. They also bind to RNA and proteins. This covalent binding can lead to cell death and mutations or transformation to a cancer cell. The binding of chemical carcinogens or their metabolically reactive products to DNA is believed to be essential in chemical carcinogenesis (Miller and Miller 1981). Therefore, epoxide hydrolase appears to play an important role in both toxication and detoxication in the cell (Guenther, 1981). In addition to its functional role in the deactivation of xenobiotics, epoxide hydrolase also has an endogeneous role in the cell.

Epoxide hydrolase is involved in the metabolism of steroids. In 1979, Bindel et al developed an assay to show that the enzyme responsible for the hydration of xenobiotics also hydrated the steroid epoxide 16 , 17 epoxy, 1,3, 5, 10 estratrien 3-ol (estroxide) in rat liver. Also, at that time they showed that testes microsomal fractions possessed a epoxide hydrolase of high specific activity.

This activity increases markedly during puberty. In 1982, the same investigators showed that rat liver microsomal epoxide hydrolase catalyze the hydrolysis of a steroid epoxide (androstine oxide) of the androgen series. The properties of the reaction was compared to those of reaction with a steroid epoxide of the estrogen series, estroxide, and two well characterized xenobiotic substrates, benzo(a)pyrene-4,5,-oxide and styrene oxide. Watabe and Akamatsu (1974) demonstrated that epoxide hydrolase plays a role in lipid peroxidation. The data indicated that lipid peroxidation of microsomes plays an important role in regulating not only the rate of epoxide formation from the olefin, but also the half life of the epoxide formed in vitro.

Purification and Properties of the Enzyme

Oesch and Daly (1971) were the first to partially purify microsomal epoxide hydrolase. The enzyme was isolated from guinea pig liver by solubilization in cutscum followed by precipitation with ammonium sulfate and desalting on a Sephadex G-25 column. A 14% yield, with a 40-fold increase in specific activity, was obtained. This basic scheme for purification with some modifications have been used by several investigators (Oesch et al, 1974; Lu et al, 1975, 1979; Levin et al, 1978; Griffin et al, 1978). However, a more rapid method of purification of the enzyme was developed by Knowles and Burchell (1977). This procedure involves deter-

gent solubilization (Lubrol-W X) and ion exchange chromatography. These techniques are reported to give a 36% yield and 460-fold purity of the enzyme. Kennedy and Burchell (1983) developed a single step method of purification of microsomal epoxide hydrolase from crude solubulized microsomes. They used monoclonal antibody to epoxide hydrolase coupled to a cyanogen bromide activated Sepharose 4B affinity column to isolate the enzyme.

The enzyme has been purified to apparent homogeneity as judged by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, immunodiffusion, centrifugal sedimentation studies, and its amino acid analysis. The physical characteristics and the biochemical and molecular properties of the enzyme have been well established.

The protein is quite hydrophobic in nature due to its membrane location and its propensity for forming large aggregates in aqueous solution. The purified enzyme(s) contains a single polypeptide of approximately 49,000 daltons in the presence of SDS. Other molecular weights of 48,000, 50,000, 53,000, 54,000, 57,000, 58,000 and 59,000 have been reported (Oesch et al, 1984; Thomas et al, 1981; Guengerich et al, 1979). In the absence of SDS, the enzyme aggregates into an oligomer with a molecular weight of approximately 600,000 (Guengerich et al 1979) which corresponds to an S-value of 14.5, which does not migrate into polyacrylamide gels (Guengerich et al 1979; Knowles and Burchell, 1977). The

differences in molecular weights for epoxide hydrolase may be due to isolation from different strains of animals (Graichen et al, 1984) or due to multiple forms of the enzyme isolated (Guengerich et al, 1979). Guenther et al (1980) purified cytosolic and microsomal epoxide hydrolase from mouse and rat liver. Antibodies to the enzymes from the two species were raised and tested. The results indicated that the two microsomal and cytosolic forms of the enzyme are immunologically the same. However, no cross reactivity was observed with the microsomal and cytosolic form from the same animal species. Guengerich et al (1979) have shown that various rat and human epoxide hydrolase(s) are immunochemically similar enzymes. The antibody preparation showed high cross reactivity with antigen from rat liver, lung, kidney and testis induced with 3-methylcholanthrene, phenobarbital and trans-stilbene oxide with human epoxide hydrolase.

Guengerich et al (1979) were the first of demonstrate that multiple forms of epoxide hydrolase exist. Utilizing ion exchange chromatography, peptide mapping, immunochemical studies, and amino acid composition they demonstrated that untreated and phenobarbital (PB)-treated rats have different forms of epoxide hydrolase from that of 3-methylcholanthrene (3-MC) treated rats, trans-stilbene oxide (TSO) treated rats and from humans. Lyman and Poland (1980) conducted crosses and backcrosses between C57BL/6J and DBA/2J strains of mice to identify the epoxide hydrolase gene. The results obtained showed genetic polymorphism of hepatic microsomal epoxide hydrolase activity among inbred mouse strains. That is, the gene locus, Eph1, codes for two allelic forms of microsomal epoxide hydrolase in

mice. Levin et al (1983) demonstrated that a different form of epoxide hydrolase was induced by cholestrol epoxide (referred to as "cholestrol-induced epoxide hydrolase") from that of the "xenobiotic induced" form (phenobarbital, SKF-525A, g-chlordane, TSO, pregnonalone 16 a-carbonitrile, isosafrole and 2-acetylaminofluorene were used as inducers). Enzymatic assays for epoxide hydrolase activity demonstrated that there is no overlapping substrate specificity between the "cholestrol-induced epoxide hydrolase" and the "xenobiotic-induced epoxide hydrolase." Immunoprecipitation studies also revealed that antibody raised to "xenobiotic EH" was not reactive with the cholesterol 5,6, - oxide hydrolase. Guenther and Oesch (1983) identified and characterized in C/57B46 mice a new microsomal epoxide hydrolase (mEH_2). They reported the enzyme catalyzes the hydration of trans-disubstituted oxiranes such as trans-stilbene oxide and trans-ethyl styrene oxide but not benzo(a)pyrene oxide, the known substrate for the microsomal form previously reported (referred to as mEH_1). Antibody to mEH_1 will not cross react with mEH_2 and the pH optima for the enzymes are different. A comparison between the new microsomal form (mEH_2) with the cytosolic form showed similarities in substrate specificities and molecular weight (Guenther and Oesch, 1983). Oesch et al 1984 isolated and purified a new form of microsomal epoxide hydrolase from Spragrue-Dawley rats and New Zealand white rabbits. They referred to the new form as cholesterol epoxide hydrolase (EH_{CH}) because

of its specificity for cholesterol 5,6 oxide, a trisubstituted epoxide. The previous form (EH_B, broad substrate specific) identified in their laboratory did not hydrate this substrate. The two enzyme forms can be discriminated by inhibitors, in that 5a ,imino-5-g-cholestane - 3B-ol potently inhibits EH_{CH} but not EH_B while 1,1,1 trichloropropane has the opposite specificity.

The pH optimum of the isolated enzyme(s) (depending on the substrate) is between 7.4 and 9.4 (Oesch, 1974; Lu 1977; and Guengerich et al, 1979). The enzyme is remarkably stable when purified to homogeneity (Bentley and Oesch, 1975). It can be stored for weeks or months at 4°C with less than 10% loss of activity (Bentley and Oesch, 1975). The absorption spectrum of the homogeneous protein has an extinction maximum at 280 nm and a small, broad but significant absorption between 300 nm and 500 nm, the latter in agreement with the faint yellow color of the enzyme solution. An absorption at 290 nm is also observed, reflecting that epoxide hydrolase contains a large amount of tryptophan (Bentley and Oesch, 1975).

Amino acid analysis indicates that the apparently homogeneous microsomal epoxide hydrolase contains relatively large amounts of tryptophan and tyrosine and a high percentage of nonoplar residues (56%) (Lu et al, 1975 and Bentley et al, 1975). DuBois et al (1979) was the first to sequence the amino acid residues of rat liver microsomal epoxide hydrolase at the N-terminal. They showed that methionine was the first amino acid in the sequence. The C-terminal sequence was found to be valine-

glutamine-alanine- OH. Bentley et al (1975) reported that the C-terminal residue was either asparagine or a glutamine residue. DuBois et al (1982) sequenced the human microsomal epoxide hydrolase and demonstrated that the N-terminal sequence is identical to that of the rat. Heineman and Ozols (1984) determined the complete amino acid sequence of hepatic microsomal epoxide hydrolase (isolated from PB treated rabbit). Their results showed that the protein contains 455 amino acid residues in a single polypeptide chain and has a molecular weight of 52,691. A comparison of the NH_2 terminal of the rabbit form of the enzyme with the human constitutive form shows that both have a hydrophobic sequence. This hydrophobic sequence is functionally the same as the "signal sequence" which is required for membrane insertion (Okada et al, 1982)

The purified enzyme can hydrate a variety of alkene oxides (such as styrene-7,8 oxide and octene 1,2 oxide), as well as the K-region arene oxides of phenanthrene, 7-methylbenzo(a)anthrene, 3 methylcholanthrene, benzo(a)anthrene and the non-K-region arene oxides of napthalene and benzo(a)pyrene (Guengerich et al, 1974; Lu et al, 1977; Bentley et al, 1976). Among the oxides tested, phenanthrene-9,10 oxide is the best substrate, whereas dibenzo(a)(h)anthracene is the poorest.

The most sensitive and widely used assay procedure is a radiometric method developed by Oesch et al (1970) using (7^3-H) styrene oxide as the substrate. Microsomes from rat, guinea pig and monkey were assayed and found capable of catalyzing the conversion of styrene oxide to styrene

glycol. A modification of this assay to a microassay to use as small as 10-20 mg biopsy specimen of human liver was developed by Oesch et al (1974). Schmassman et al (1975) modified the microassay to a more rapid procedure. Using the K-region epoxide of the potent carcinogen benzo(a)-pyrene as a substrate, they succeeded in finding conditions in which the unreacted substrate can be separated from the product by simple extraction. In addition to the radiometric assays, spectrophotometric (Watabe and Akatmsu, 1974; Hasegawa and Hammock 1982; Craven et al, 1982; Westkaemper and Hanzlik, 1981), fluorometric (Dansette et al, 1979), high performance liquid chromatographic (WestKaemper and Hanzlik, 1980) and gas chromatographic assays (Westkaemper and Hanzlik, 1981) are used.

Distribution and Location

According to Walker et al (1978) liver microsomal epoxide hydrolase activity is found in a wide variety of vertebrate species. These studies were carried out with hexachloro-6,7-epoxy1,4,4a,5,6,7,8,8a-octa hydro-1, 4 methananapthaline (HEOM), benzo(a)pyrene-4,5 oxide and styrene 7,8 oxide as substrates. All three of these substrates are hydrated by microsomal epoxide hydrolase in all species investigated. Their results showed that larger mammals have the highest activity for the enzyme and that rodents, birds, amphibians and fish have less activity in the descending order listed. Epoxide hydrolase is also found in other organisms. Brooks (1973) showed that flies, roaches and beetles contain epoxide

hydrolase activity. Banthorpe and Osborne (1984) have isolated the enzyme from plant tissue. The enzyme was found in extracts of both leaves of Tanacetum vulgare (tanzy) and in callus cultures of Jasminum officinale-L.

Early studies done by Oesch et al (1970, 1971, 1974) established that the enzyme is primarily found in the endoplasmic reticulum in close association with the cytochrome P-450 system. Based on its universality in the endoplasmic reticulum, Gill and Hammock (1980), Oesch (1974) and Griffin and Gengozian (1984) have suggested the use of epoxide hydrolase as a marker enzyme for the microsomal membrane and heptocarcinogenesis. Investigations done by Mukhtar et al, (1979) Gontonvick and Bellward (1981) and Pacifici et al (1984) have also demonstrated that epoxide hydrolase is present in the cell nuclei, mitochondria and cytosol. Other organelles that contain epoxide hydrolase activity are the golgi and plasma membrane (Stasiecki, 1981).

The liver has been the main organ for isolation of epoxide hydrolase and it contains the highest activity for the enzyme. However, Gill et al (1979) and Lu et al (1980) have isolated the enzyme from the ovary, lung and the kidney and showed that these organs contain activity for epoxide hydrolase equal to that of the liver. Epoxide hydrolase is also found in the tracheae, skin, and tongue (Oesch et al 1977; Bickers et al 1984) but in lower concentration than in the former organs mentioned. More recently, Seidegard et al (1984) have demonstrated that the enzyme is found in blood cells (leukocytes).

Membrane Topology

Lu et al (1975) and Seidegard et al (1978, 1982) investigated the lateral topology of epoxide hydrolase in the membrane of the endoplasmic reticulum by subfractionation of microsomes. Separation into rough and smooth microsomes revealed a slightly higher level of epoxide hydrolase in the rough microsomes and that the enzyme is not randomly distributed along the endoplasmic reticulum membrane. Studies done by Guengerich and Davidson (1982) evaluated the interaction of microsomal epoxide hydrolase and other proteins in situ in the membrane and concluded that microsomal epoxide hydrolase forms a complex with certain isozymes of cytochrome P-450. Seidegard et al (1982) used ^{125}I in the presence of lactoperoxidase to study the transverse topology of microsomal epoxide hydrolase. The results suggest that 20-25% of the polypeptide is exposed at the cytoplasmic surface of the endoplasmic reticulum and that the enzyme is not exposed at all at the luminal surface.

Regulation and Biosynthesis

Hepatic epoxide hydrolase activity increases with maturation in mammals and sex-linked differences in activity are seen (Oesch 1976; Batt et al, 1984). During fetal development, epoxide hydrolase becomes measurable only during the last four days before birth, steadily increasing thereafter. Enzymatic activity is barely measurable in livers of neonatal rats while livers from adult male rats have from 2.5 to 3 times the

specific activity found in the livers from adult females (Sharma et al, 1979; Hammock et al, 1983; Parkinson et al, 1983; Waechter et al, 1984).

Epoxide hydrolase activity may be elevated by induction. Oesch et al (1971) found that epoxide hydrolase reaches maximal levels during maturation of rats and that these levels can be increased by pretreatment of animals with PB or 3-MC. Their data suggested that the induced enzyme is substrate specific and that there is more than one epoxide hydrolase in liver microsomes. Bucker et al (1979) showed that trans-stilbene oxide (TSO) is a potent inducer of epoxide hydrolase in rat liver. Animals received intraperitoneal injections of 2 moles per kilogram of body weight of TSO on three consecutive days. Following the injections, the rats showed a 3-fold increase in enzyme activity. Thomas et al (1981) compared the ability of corn oil, pregnenoline 16-a-carbonitrile, TSO, SKF-525A, g-chlordane, morphine, PB, 3-MC, Aroclor 1254 and isosafrole to induce epoxide hydrolase in immature and mature rats. They found that morphine, corn oil and SKF-525A have little or no effect on levels or catalytic activity of the enzyme. Treatments with the other xenobiotics produced significant increases in both enzyme concentration and catalytic activity with the greatest induction by TSO. It was also observed that the ratio of catalytic activity of the enzyme concentration is constant despite the wide variation in epoxide hydrolase levels after treatment with these xenobiotics. Seidegard and DePierre (1980) showed that benzil is a potent activator of microsomal epoxide hydrolase in-vitro. Cells

from liver of rats injected intraperitoneally with benzil were used in this investigation. Their results showed a 9-fold increase in both the apparent V_{max} and Km of the enzyme. This activation was noncompetitive, suggesting that the activator does not bind to the same substrate, styrene oxide. Kuhlman et al (1981) showed that treatment of rats with N-nitrosomorpholine (NNM) for seven weeks led to a focal increase in liver microsomal epoxide hydrolase as early as two weeks after withdrawal of the carcinogen. To see if these levels were reversible, livers were examined at 2, 6, 12, 18 and 26 weeks after discontinuation of NNM feeding. The elevation of epoxide hydrolase persisted throughout the entire observation period and the focal increase was seen at very early stages in hepatocarcinogenesis. The following stages, including benign hepatomas, were high in enzyme activity but this activity was not observed in the malignant tumors investigated. Astrom and DePierre (1981) observed that the treatment of rats with 2-acetylaminofluorene (2-AAF) once daily for five consecutive days increased the levels of epoxide hydrolase greater than 7-fold even though 2-AAF is thought not to be metabolized by epoxide hydrolase. It was also observed that TSO induction was more than 700% of the control values.

Despite the recent advances in the purification and characterization of rat liver epoxide hydrolase, only recently have any studies focused on the capacity of mRNA to direct the synthesis of epoxide hydrolase in an in vitro system. Gonzales and Kasper (1980) were the first to synthesize

epoxide hydratase (hydrolase) *in vitro* in a rabbit reticulocyte lysate translational system. Messenger RNA was isolated from membrane-bound polysomes and free polysomes of phenobarbital treated rats to use in the cell-free system. Their results showed that only mRNA isolated from membrane-bound polysomes coded for the synthesis of epoxide hydrolase. The molecular weight and partial proteolytic peptide patterns of the *in vitro* synthesized EH was identical to that of the native enzymes indicating the absence of a cleavable signal sequence. Pickett and Lu (1981) synthesized epoxide hydrolase in a cell-free system and confirmed that mRNA isolated from membrane-bound polysomes synthesized the enzyme. Their findings also suggested that, unlike many secreted and some membrane proteins, epoxide hydrolase appears not to require a cleavable signal sequence for insertion into the endoplasmic reticulum. Ohlsson *et al* (1981) used *Xenopus laevis* (frog) embryo membranes to show the synthesis and insertion of epoxide hydrolase *in vivo* and *in vitro*. Microinjection of rat liver RNA into whole oocytes suggested that membrane insertion is neither cell-type nor species specific because rat liver epoxide hydrolase can be inserted into the endoplasmic reticulum of the frog oocyte membranes. The association is highly selective since, unlike albumin and at least 15 other rat liver proteins, epoxide hydratase (hydrolase) is not secreted in detectable amounts. Less than 5% of the epoxide hydrolase inside the

oocyte is found in the medium. Okada et al (1982) synthesized epoxide hydrolase in a wheat germ extract cell-free system which was programmed with poly(A)-containing mRNA extracted from free and membrane-bound polysomes of rats pretreated with 3-methylcholanthrene. Epoxide hydrolase mRNA was found only on bound polysomes and the product of its translation in vitro had the same electrophoretic mobility as the mature protein, suggestng that epoxide hydrolase is not processed by removal of an amino-terminal insertion signal.

CHAPTER III

MATERIALS AND METHODS

In Vivo Isolation Of Epoxide Hydrolase

Induction Of Epoxide Hydrolase

Adult male (Long-Evans strain) rats weighing from 250 to 300 mg were used. They were divided into two groups (five rats per group): the control and the styrene oxide (SO) treated groups. Rats were housed in a regular laboratory animal facility and fed a regular rat chow pellet diet throughout the experiments. Both groups were treated for 48 hr. They received intraperitoneal injections every 24 hr and were fasted 24 hr prior to sacrifice. The control group received one milliliter of pure corn oil (vehicle) and the SO treated group received 500 mg of styrene oxide (Aldrich Chemical Company) dissolved in one milliliter of the vehicle. Forty-eight hours post-injection, both groups were lightly anesthesized with ether and sacrificed by guillotining.

Isolation Of Smooth and Rough Microsomes and Free Poly ribosomes Of Styrene Oxide Treated Rats

The rat livers were excised and quickly removed, blotted on aseptic absorbent paper towels, weighed and cut into small pieces. The tissue was placed in an ice cold (0-4^{o}C) 0.25M homogenization buffer (85% sucrose, 1M triethanolamine, 1M potassium chloride, 1M magnesium chloride - referred to hereafter as STKM) in preparation for fraction-

ation (Adelman et al, 1973). The tissue was homogenized with a Teflon pestle-glass homogenizer, size C, rotating at 2000 xg. The homogenate was centrifuged at 8000 xg for 5 min at 0°C in a Beckman J2-21 model centrifuge to obtain the post-nuclear supernatant (PNS). The PNS was centrifuged at 12000 xg for 10 min at 0°C in a Beckman J2-21 model centifuge to obtain the post-mitochondrial supernatant (PMS). The PMS was fractionated on a discontinuous sucrose density gradient to obtain the smooth microsomes (SM), the rough microsomes (RM) and the free polyribosomes (FP). The discontinuous step gradient contained concise regions of 1.3M, 1.5M and 2.0M STKM. The overlayered PMS preparations were centrifuged at 120,000 xg for 24 hr at 0°C in a Beckman L8-70 model ultracentrifuge to sediment the aforementioned fractions. The SM, RM and FP preparations were carefully removed from the gradient layers and treated in the following manner. The FP fraction was immediately resuspended in 1 ml of sterile distilled water and aliquoted into 20 µl amounts. The 20 µl aliquots of the FP fractions were quickly frozen in liquid nitrogen and stored at -80°C until ready for use in the in vitro translation experiments. The remaining portion of the RM and the SM fractions were carefully layered on cushion gradients consisting of 1.3M and 2.0M STKM, respectively, and centrifuged at 120,000 xg at 0°C for 1 hr to reduce contamination with other membranes. The SM was carefully removed from the cushion gradient and mixed with an equal volume 0.25M STKM and centrifuged at 120,000 xg at 0°C for 1 hr to pellet the SM.

The RM was also removed from the cushion gradient prior to pelleting and was quickly frozen in liquid nitrogen and stored at -80°C for *in vitro* translation experiments. Protein concentrations of each fraction were determined spectrophotometrically at 280 nm and and the RNA concentration of the RM and FP was determined at 260 nm spectrophotometrically (Fleck and Munro, 1962) in a Beckman DU-8 model spectrophotometer.

Spectrophotometric Assay For Epoxide Hydrolase Using Trans-Stilbene Oxide As The Substrate

The spectrophotometric assay for epoxide hydrolase developed by Hasegawa and Hammock (1981) was used to determine the enzymatic activity of the styrene oxide induced form of epoxide hydrolase (EH_{SO}). All ultraviolet spectra were performed at 37°C on a Beckman DU-8 model spectrophotometer. All spectral measurements were made in 0.2M potassium phosphate (K_2PO_4) buffer, pH 7.4. Epoxide hydrolase activity was assayed in a final volume of 2 ml. The 2 ml incubation mixture consisted of.05-2 mg of microsomal protein, .02M TSO, .04M KPO_4, pH 7.4, and dH_2O. The buffer was equilibrated in the sample and reference cuvettes for 5 min. at 37°C. Ice-cold microsomal preparations (50-200 μg) from the styrene oxide treated rats or the control rats were added to both cuvettes, and the spectrophotometer was optically balanced. Forty microliters of 100% ethanol was placed in the reference cuvette. The reaction was initiated

by the addition of 40 μl of 0.25M trans-stilbene oxide (TSO) in ethanol with thorough mixing. The reaction was monitored at 229 nm at 30 sec intervals for 5 min.

Partial Purification of Microsomal Epoxide Hydrolase From Styrene Oxide Treated Rat Liver

Microsomal epoxide hydrolase was partially purified using a modification of the method of Knowles and Burchell (1977). All purification steps were performed at 4°C. Smooth and rough microsomal pellets were solubilized in 0.2MKPO_4/1% lubrol buffer, pH 7.0 (buffer A). The solubilized pellets were dialyzed overnight against 4 liters of 5mM KPO_4/0.05% lubrol buffer, pH 7.4 (buffer B). The dialysate was lyophilized and resuspended in 0.5 ml of buffer B and applied to a 40cm X 2cm DEAE cellulose column and eluted with buffer B. Collected fractions were assayed for epoxide hydrolase activity. Fractions containing epoxide hydrolase activity were pooled and dialyzed against 4 liters of 5mM KPO_4/0.05% lubrol buffer, pH 6.5 (buffer C). A 200 μl aliquot of this dialysate was quickly frozen in liquid nitrogen and stored at -80°C for use in Concanavalin A-binding studies. The remaining volume of the dialysate (31 ml) was lyophilized and resuspended in 0.5 ml of buffer C and applied to a 20cm X 1.5cm Carboxymethyl (CM) cellulose column. Fractions containing epoxide hydrolase activity were pooled and quickly frozen in liquid nitrogen and stored at -80°C.

Preparation of Proteins For SDS Polyacrylamide Gel Electrophoresis

Protein samples (20 to 50 µl volumes) containing epoxide hydrolase activity (7.5 to 25 µg of protein) were precipitated with ice cold 10% trichloracetic acid (TCA) for 30 minutes at 4°C. The samples were centrifuged at 18,000 xg for 2 minutes in a Eppendorf 5412 model microfuge to pellet the protein. The pellet was resuspended in a gel loading buffer (0.02% bromophenol blue, 200mM EDTA, 42.5% sucrose and 0.1M tris base, [referred to hereafter as BEST soln], and 1.0M DTT and 20% SDS [referred to as DS]) and sonicated with a Heat Systems sonicator model 220 at 20 decibles per min for a total of 30 sec. The BEST-DS treated samples were incubated in a 37°C water bath for 30 minutes for solubilization. The solubilized samples were boiled for 2 min, cooled to room temperature and 10 µl of 0.25M indoacetimide was added. The samples were re-incubated in a 37°C water bath for 20 min and loaded on a gel.

SDS-Polyacrylamide Gel Electrophoresis Analysis Of The Partially Purified Styrene Oxide Induced Epoxide Hydrolase

Microsomal preparations and DEAE cellulose column and CM cellulose column eluates containing epoxide hydrolase activity were analyzed on 7.5% sodium-dodecyl sulphate (SDS) polyacrylamide gels. The samples (7.5 - 25 µg of protein) were prepared for electrophoresis according to Laemmli (1970) and followed by alkylation using 0.25M indoacetimide.

Samples were electrophoresed for 17 hr at 60V and 1.5 milliamps per slot in a discontinuous buffer system. The gels were stained and destained according to Laemmli (1970). The molecular weight of epoxide hydrolase was determined by comparison of the measurement of its relative mobility to the measurement of molecular weight standards relative mobilities in a SDS-polyacrylamide gel (Weber and Osborn, 1969).

Concanavalin -A- Sepharose Affinity Chromatography Analysis of Epoxide Hydrolase

Concanavalin -A- sepharose affinity chromatography was performed to determine if EH_{SO} was glycoprotein. Procedures used were a modification of the methods of Kennedy and Rosevear (1973). A 200 μl volume (150 μg of protein) of the dialysate from the DEAE cellulose column was applied to 7cm X 0.7cm column containing concanavlin -A- sepharose beads equilibrated in a 5mM KPO_4/0.05% lubrol buffer, pH 7.4. The unbound fractions were eluted with the equilibration buffer. The column was washed 3X with equilibration buffer. The bound fractions were eluted with the alpha-methyl mannoside. Fractions were analyzed on a 7.5% SDS-polyacrylamide gel.

Gel Double Immunodiffusion Analysis Of Epoxide Hydrolase

Rabbit anti-fish epoxide hydrolase [49000 dalton molecular weight] was a gift from Nathan Jideama. Ouchterlony's immunodiffusion technique was done to test antigen antibody reactions (Ouchterlony, 1949). The test medium consisted of 2% agarose made in 0.8% borate-buffered saline,

pH 7.5 containing 0.01% merthiolate and 0.002% trypan blue. The test medium was poured 5mm to a height in a 65mm X 15mm ($25cm^3$) petri dish and allowed to solidify at room temperature. Ten to twenty microliter (25-50 μg) of epoxide hydrolase was placed in the outer wells and 20 μl (50 μg) of anti-serum to epoxide hydrolase was aliquoted into the center well. The plates were incubated at 37°C in a moist chamber for 1-3 days. They were monitored for the formation of antigen-antibody precipitin lines every 24 hr.

RNA Extraction From Styrene Oxide Treated Rat Liver

Total liver RNA was isolated from styrene oxide treated rats 16 hr post-injection. Extraction was done according to the methods of Sherrer and Darnell (1962). Liquid nitrogen frozen liver was ground into a fine powder which was mixed with extraction buffer (0.05M Tris, pH 7.5, 0.005M EDTA, pH 7.0, 0.15M NaCl, 5% SDS) consisting of an aqueous phase and an organic phase: phenol: chloroform: isoamyl alcohol (50:50:1) and blended in a Waring blender for 30-60 sec. The mixture was stirred for 10 min, centrifuged at 3000 xg for 10 min at 12°C. The total RNA was precipitated at -20°C with 100% ethanol and 2.5M lithium chloride. The final acquired RNA pellet was dissolved in 5 ml of sterile distilled water. An O.D. reading at 260 nm was done to determine the concentration in A_{260} units (Fleck and Munro, 1962). The sample was aliquoted

into 50 µl volumes and quickly frozen in liquid nitrogen and stored at -80°C. A RNA titration curve was done to determine the amount of RNA most efficient in the synthesis of proteins in an in vitro rabbit reticulocyte translational system.

In Vitro Isolation Of Epoxide Hydrolase

Cell Free Synthesis of Epoxide Hydrolase

Incorporation of [^{35}S]-methionine into protein was performed in a rabbit reticulocyte lysate cell-free translational system. In vitro translation systems consisting of nuclease-treated rabbit reticulocyte lysate and support reagents, programmed with total RNA or RER-MBP or free polysomes were processed after the methods of Pelham and Jackson (1976). Nascent polypeptides were synthesized ina 25 µl translation mixture consisting of 12.5 µl of lysate (Promega Biotec Lot M116V), 3-5 µl of total RNA, 5 µl of energy mix (0.1M ATP, 0.02M GTP, 0.6M creatine phosphate (CP), 1mM 19 amino acids, [^{35}S]-methionine [5.25 mCi/0.31], 1M KOH, 8 mg/ml creatine phosphokinase); 5 ul compensation buffer (1M Tris-HCl, pH 7.4, 4M KCl, 1M $MgCl_2$, distilled H_2O) and 0.2µl of distilled water. The reaction was incubated at 29°C for 59 min.

Co-Translational Insertion and Post-Translational Proteolysis of The In Vitro Synthesized Products

Dog pancreatic microsomal membrane (DPM) was added co-translationally to *in vitro* translation reactions that contained total RNA or free polysomes. Trypsin (80 μg/ml) and chymotrypsin (80 μg/ml) were added post-translationally to *in vitro* translation reactions that contained total RNA, or RER-membrane-bound polysomes or free polysomes. Tryptic digestion was carried out for 3 hr at 0°C. The digestion of the translational products was stopped by the addition of 30 μl trasylol (protease inhibitor) to the translation mixture.

Assay for Incorporation Of [^{35}S]-Methionine Into Proteins

To assess the incorporation of [^{35}S]-methionine into nascent polypeptides, 5 μl of the translation mixture was spotted onto a Whatman 3MM absorbant filter disc and the disc was placed into chilled 10% trichloroacetic acid (TCA) for 10 min. The filter disc was incubated in 5% TCA at room temperature for 5 min, followed by boiling in fresh 5% TCA for 10 minutes. After TCA precipitation, the disc was saturated with ethanol/ether, 50:50 (vol/vol) for 15 minutes, the ethanol/ether was aspirated off the disc and the disc was placed in ethyl ether for 15 min. Subsequently, the ethyl ether was aspirated off and the disc was air dried and placed in 10 ml of scintillation fluid (New England Nuclear liquifluor) and counted. Samples were counted in a Beckman LS 7500 model scintillation counter at an efficiency of approximately 29% for ^{35}S.

Immunoprecipitation Of Epoxide Hydrolase From Translation Products

Epoxide hydrolase was immunoprecipitated from the *in vitro* translation products according to the methods of Goldman and Blobel (1978). The *in vitro* translation products (IVT) were preabsorbed to a protein A-Sepharose ligand *in situ*. The supernatant was collected and boiled for 2 min in 2% SDS/trasylol. The mixture was diluted four-fold with 1.25% triton-X buffer containing 1M tris-HCL (pH 7.4), 2M NaCl, 0.2M EDTA and 20% triton X-100. Five microlitiers of monospecific rabbit anti-fish-epoxide hydrolase antiserum was added to the sample. The sample was centrifuged at 8000xg for 30 seconds and incubated for 1 hour at 37°C. Twenty microliters of protein A-Sepharose was added to the sample and was incubated for 1 hr at room temperature (29°C) and for 12 hours at 4°C on a BBL tube rotator. The protein A-Sepharose antigen-antibody complex was centrifuged at 8000 xg for 2 minutes. The sepharose beads were pelleted and washed four times in 1% triton-X buffer. To the final pellet, 50 μl of 4% SDS was added and the suspension was vortexed, boiled for 3 min, cooled and centrifuged at 8000 xg for 2 min. The sample radioactivity was determined by spotting a 1 μl aliquot of the immunoprecipitate on a filter disc which was counted in 10 ml of toluene based scintillation fluid (aquafluor) in a Beckman LS 7500 scintillation counter. The sample was analyzed for immunoprecipitable epoxide hydrolase by SDS-polyacrylamide gel electrophoresis.

Autoradiography Of The Immunoprecipitable Epoxide Hydrolase

The SDS-polyacrylamide gels of the immunoprecipitated epoxide hydrolase (in vitro synthesized) were dried under vacuum overnight and exposed to Kodax RP-X-O-Mat x-ray film for 72 hr at -80°C using a Cronex intensifying screen. The film was developed in an automatic x-ray film processor for visualization of synthesized (labeled) products (modified procedures of Bonner and Laskey, 1974).

CHAPTER IV

RESULTS

Epoxide Hydrolase Induction Studies

Treatment of adult male rats with 1000 ml of styrene oxide for 48 hr caused a marked increase in the total protein concentration of the endoplasmic reticulum (Table 1). The SO treated SM membrane total protein concentration was 64% higher than the control SM membrane. The RM membrane protein concentration of the SO treated rats was 156% higher than the control RM membrane protein concentration. Table 1 and Fig. 1 show the marked differences in the proliferation of the endoplasmic reticulum protein concentration of the SO treated rat liver. The SO treated fractions also showed an increase in epoxide hydrolase activity. The SO treated microsomes (SM and RM) exhibited 20% increase (34.00 U/ml) in epoxide hydrolase activity in comparison to the control microsomes (28.30 U/ml). SDS-polyacrylamide gel protein profiles also reveal an increase in the concentration of epoxide hydrolase in the SO treated microsomes (Figs. 2a and 2b).

Table 1 - The effects of styrene oxide on the protein concentration of rat liver microsomes

	Microsomes	OD_{280}	Protein Conc.* (mg/ml)
Control			
	SM	0.586 ± .008	0.685 ± .001
	RM	0.186 ± .031	0.199 ± .006
SO Treated			
	SM	0.971 ± .009	1.129 ± .003
	RM	0.430 ± .008	0.473 ± .006

*Based on A_{280} protein assay standard curve using bovine serum albumin as the standard.

Fig. 1. A graph showing the effects of styrene oxide on the induction of protein in rat liver microsomes.
Marked increases in the protein concentration of SO treated is observed. The percentage of microsomal protein concentration induction is expressed as a percentage of protein concentration of the control.

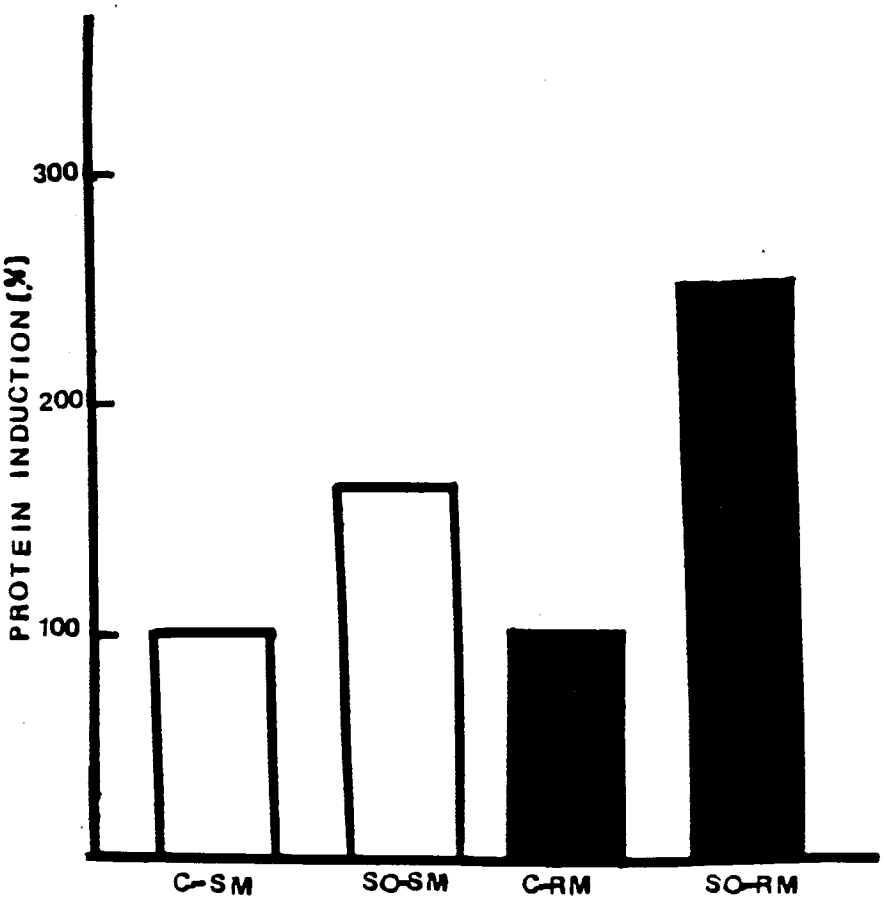
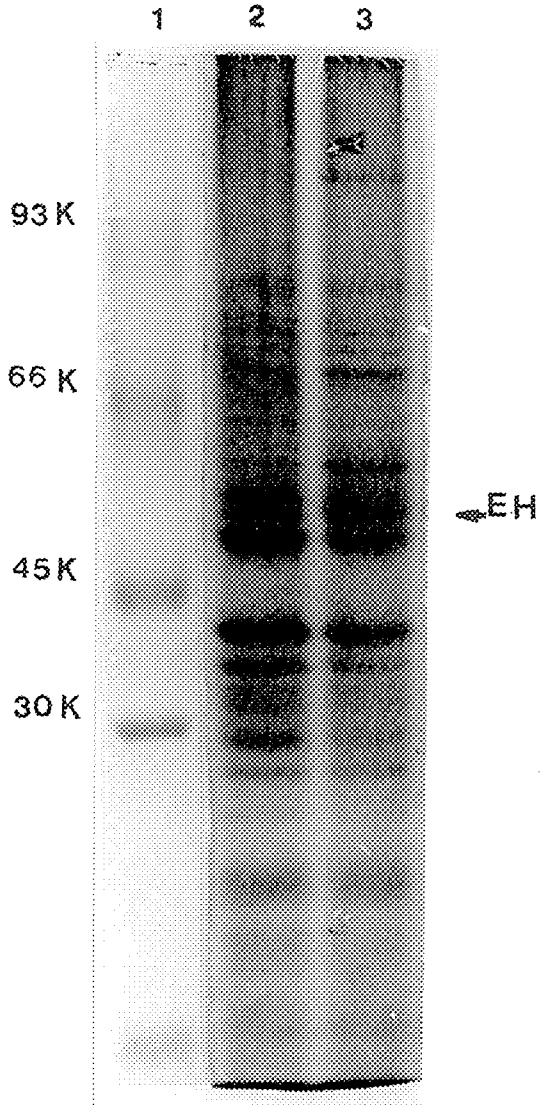

PROTEIN INDUCTION (%)
300
200
100
C-SM
SO-SM
C-RM
SO-RM

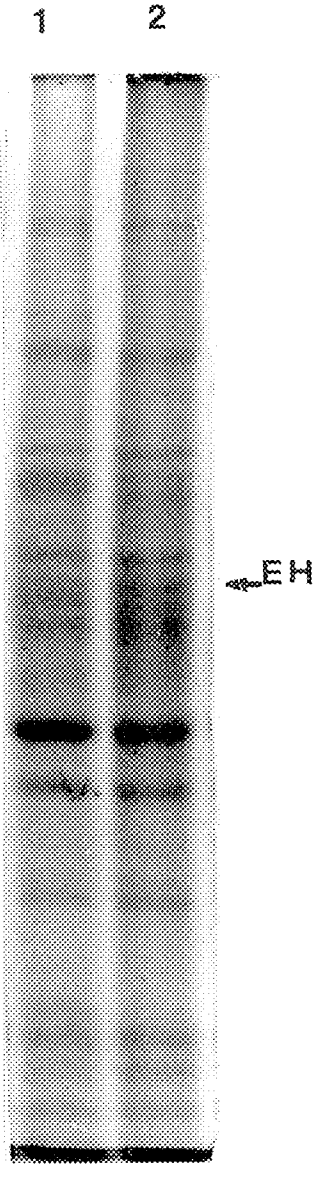
Fig. 2. SDS-polyacrylamide gel protein profiles of SO treated rat liver microsomes showing the induction of epoxide hydrolase. Samples were electrophoresed on 7.5% gels. Each lane contains 25 µg of protein.

Gel 1 - Smooth microsomes protein profile. Lane 1-molecular weight protein standards; phosphorylase b, albumin, ovalbumin, carbonic anhydrase, a-lactoalbumin; Lane 2 - control (untreated) SM; Lane 3 - SO treated SM. Arrow indicates epoxide hydrolase.

Gel 2 - Rough microsomes protein profiles, Lane 1 - control SM; Lane 2 - SO treated RM. Arrow indicates epoxide hydrolase.



1



2

Spectrophotometric Assay for Epoxide Hydrolase

The hydration of trans-stilbene oxide (TSO) by epoxide hydrolase was measured by following the decrease in absorbance at 229 nm. A typical time course for the standard assay (50 µg of liver microsomal enzyme solution, 0.25M TSO) is shown in Fig. 3. The decrease in absorbance was linear for more than 5 min. Hydration of TSO by epoxide hydrolase was linear with protein samples in the range of 50 µg to 200 µg and with time for at least 5 min. Use of the boiled enzyme (30 min at 100°C) in the assay causes no hydration of TSO.

Partial Purification of Microsomal Epoxide Hydrolase (EH_{SO})

A styrene oxide induced form of rat liver microsomal epoxide hydrolase partially purified by detergent (Lubrol-WX-Sigma Grade) solubilization and ion exhange chromatography. The enzyme was purified 130 fold from three whole rat livers. A 10% yield of EH_{SO} was obtained, exhibiting a specific activity of 382.10 units/ml of protein (see Table 2) as determined by the method of Hasegawa and Hammock (1981). SDS-polyacrylamide gel analysis of the protein revealed a single polypeptide (Figs. 4,5, & 6). Comparison of EH_{SO} with protein standards of known molecular weight showed the subunit molecular weight to be 56000 (Fig. 7).

Fig. 3. A graph showing enzymatic activity of microsomal epoxide hydrolase was linear with time.

Enzymatic hydration of trans-stilbene oxide was monitored at 229 nm. TSO was incubated with 50 µg of RM or SM from SO treated rat liver.

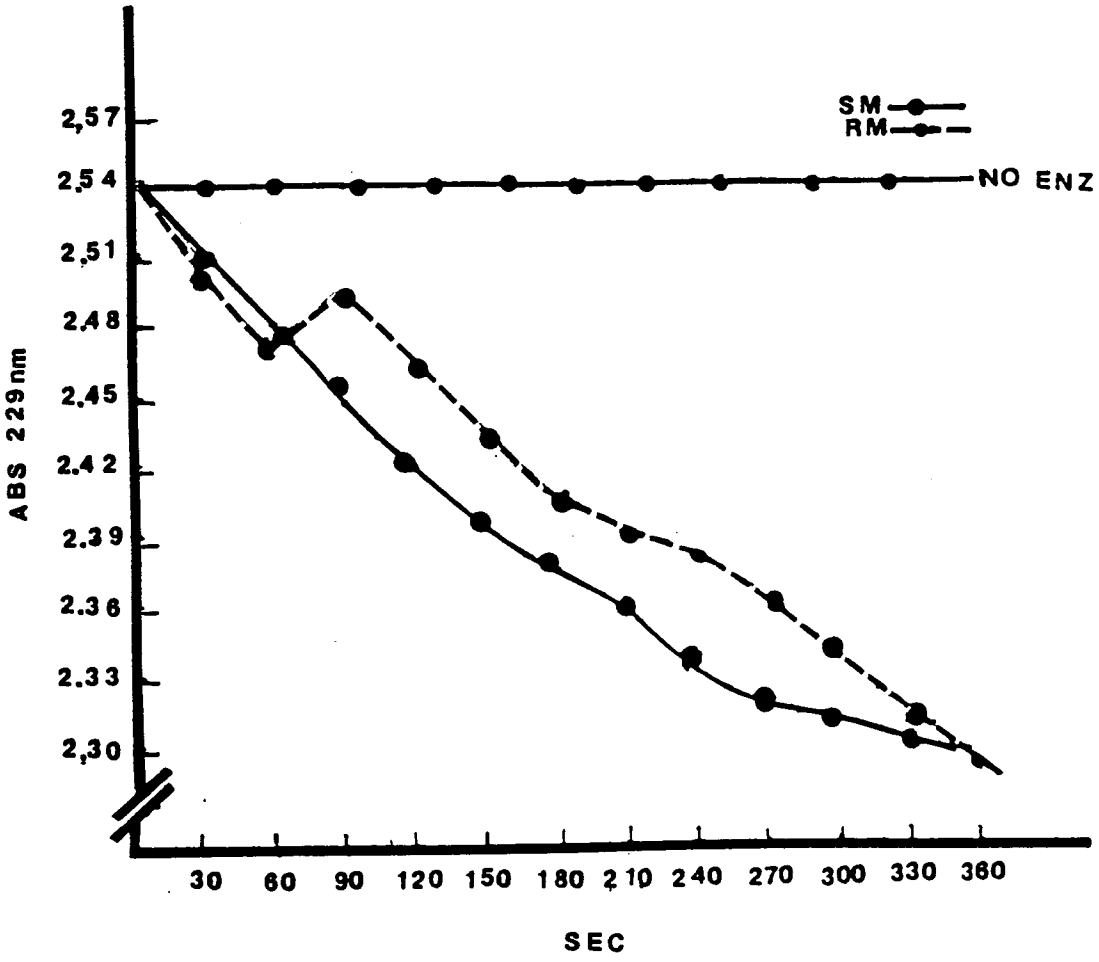

SM
RM
NO ENZ
2,57
2,54
2,51
2,48
2,45
2,42
2,39
2,36
2,33
2,30
ABS 229nm
30
60
90
120
150
180
210
240
270
300
330
360
SEC

Fig. 4. SDS - PAGE protein profile of the isolation and partial purification of EH_{SO}.

Samples were electrophoresed on a 7.5% gel.

This gel represents the isolation of the enzyme by subcellular fractionation and detergent solubilization.

Lane 1 - molecular weight protein standards;

Lane 2 - PMS (25 μg); Lane 3 - SM (25 μg);

Lane 4 - RM (25 μg); Lane 5 - Combined microsomes (25 μg); Lane 6 - Lubrol solubilized microsomes (25 μg); Lane 7 - Combined DEAE eluates (25 μg).

Arrow indicates epoxide hydrolase.

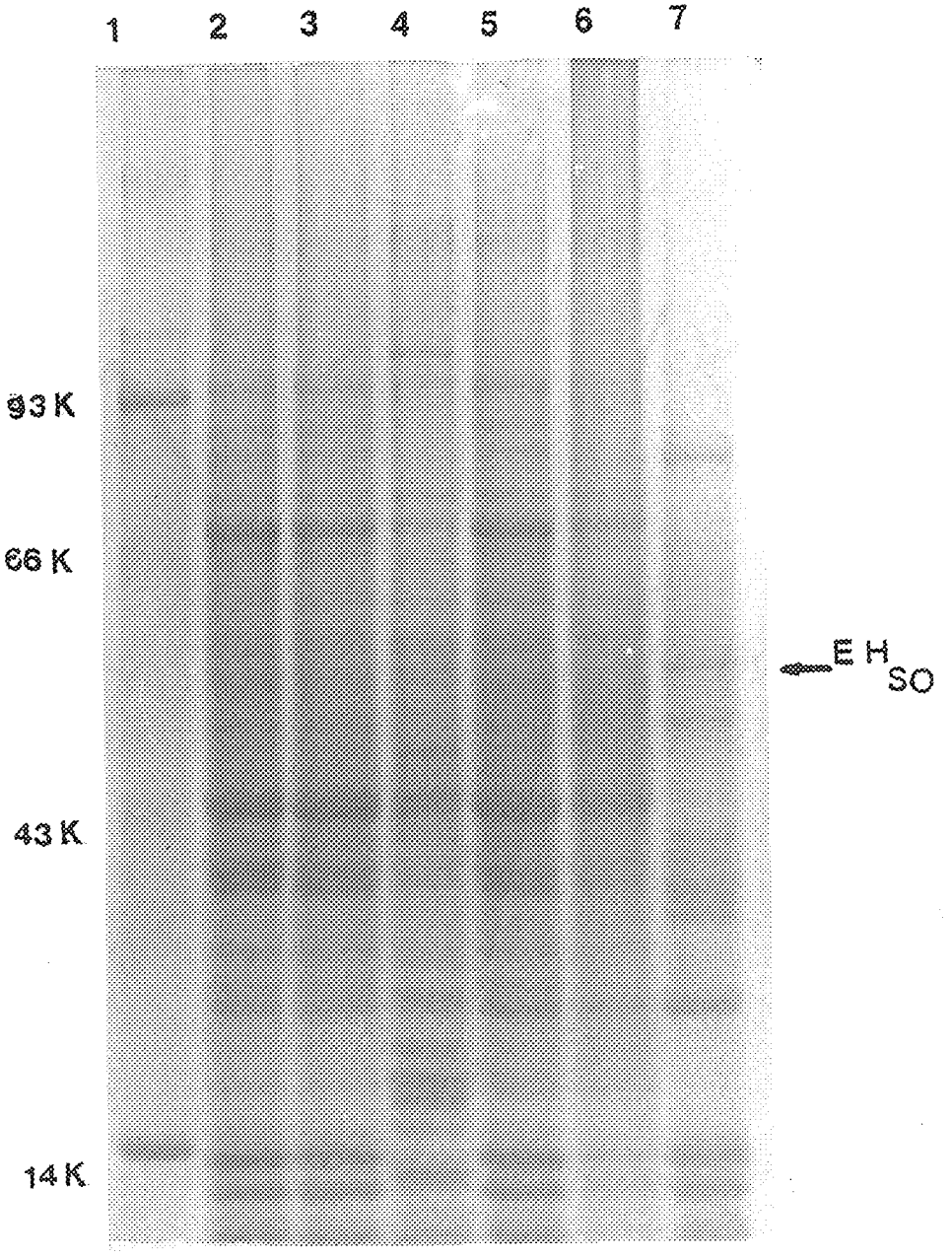

1
2
3
4
5
6
7
93 K
66 K
43 K
14 K
E H SO

Fig. 5. DEAE cellulose chromatography of solubilized microsomes containing epoxide hydrolase activity.

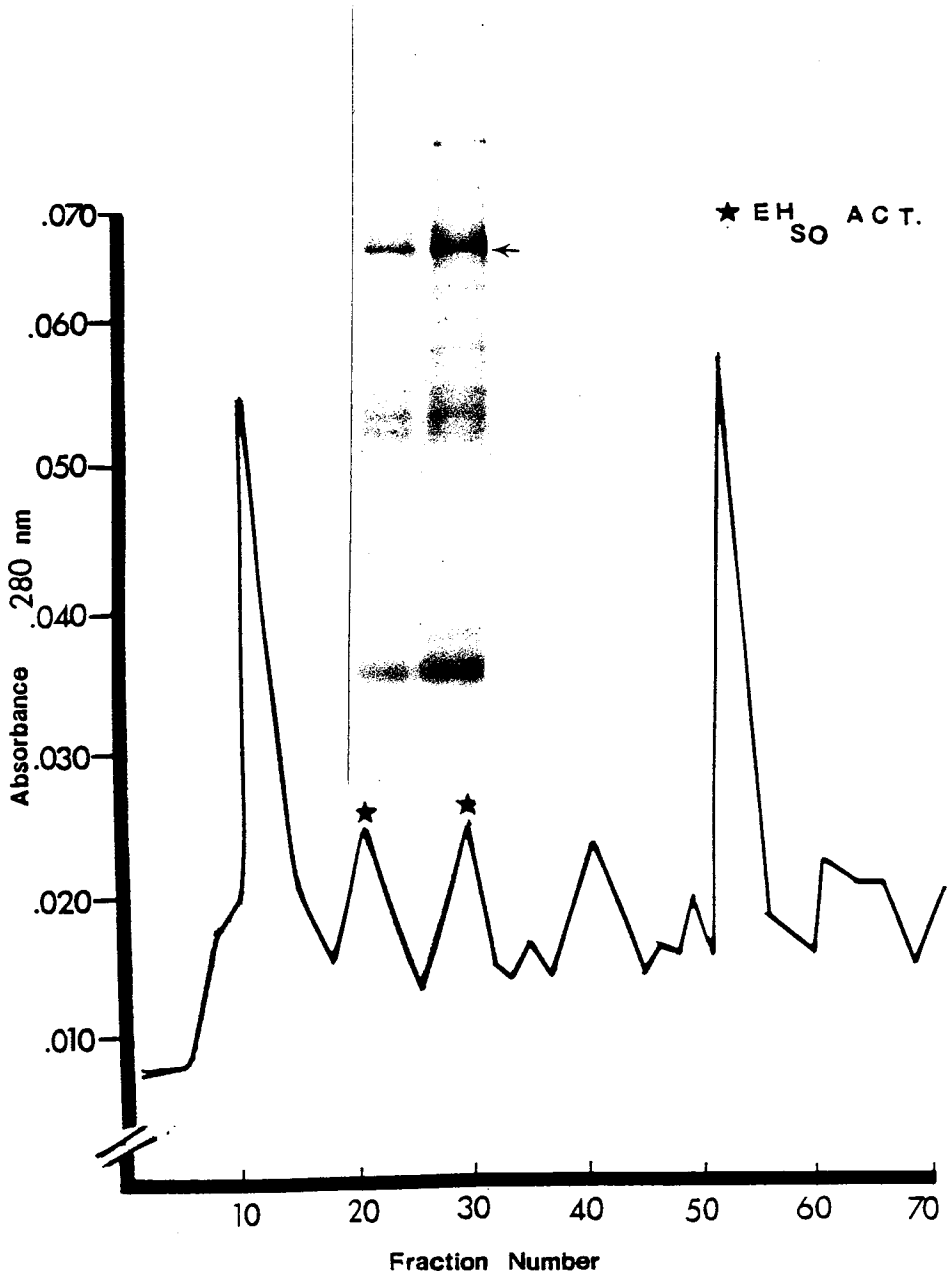
Inset - SDS - PAGE of EH_{SO}

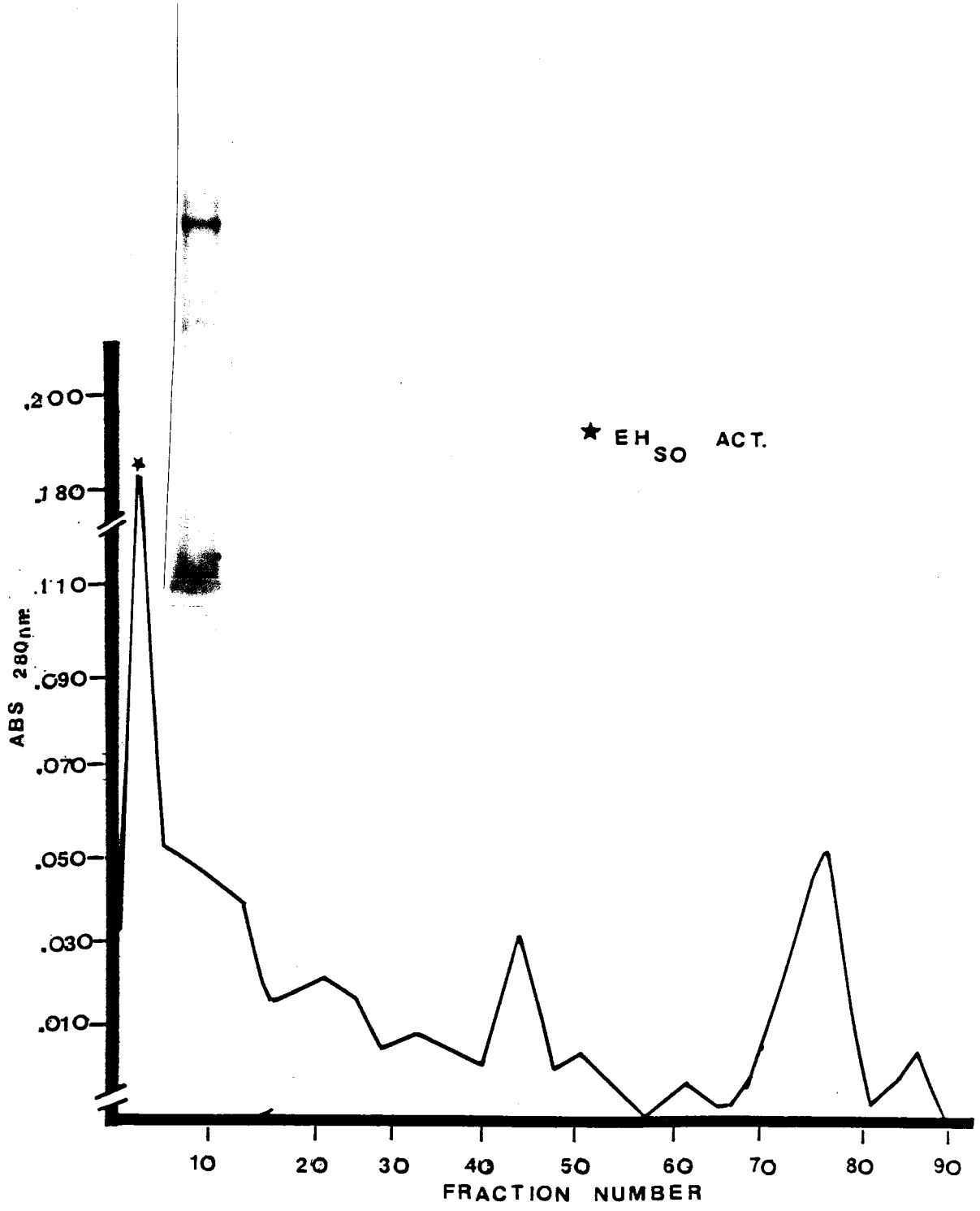
Lane 1 - DEAE eluted peak no. 2

profile; Lane 2 DEAE eluted peak no. 3.

Arrow indicates epoxide hydrolase protein band.

Chromatogram of DEAE eluted peaks with epoxide hydrolase activity. Asterick indicates peaks with EH activity.


★ EH SO ACT.
.070
.060
.050
.040
.030
.020
.010
Absorbance 280 nm
10
20
30
40
50
60
70
Fraction Number


★ EH SO ACT.
ABS 280nm
.200
.180
.110
.090
.070
.050
.030
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30
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50
60
70
80
90
FRACTION NUMBER

Table 2. Partial purification of SO induced rat liver epoxide hydrolase Data based on the purification of EH_{SO} from adult male rat livers. Average body weight was 285 grams and the livers average weight was 9.87 grams.

Purification Step	Total Protein (mg)	Specific *Activity	Relative Purification	Total Activity	Yield[%]
Cell Homogenate	1012.00	2.94	1	2975	100
PMS	340.00	8.64	3	2938	99
Crude Microsomes (SM and RM)	108.60	12.28	4	1337	45
Lubrol solubilized Microsomal Pellets	52.00	28.40	9	1477	50
DEAE Cellulose eluates	10.00	120.37	41	1204	40
CM Cellulose eluates	0.80	382.10	130	306	10

*Specific activity = U/ml = mMoles/min x ml. It is calculated on the basis of the molar extinction coefficient of the reaction substrate (TSO).

Figure 7 - SDS-PAGE analysis of the partially purified EH_{SO}.

Lane 1 - Molecular weight marker;

Lane 2 - EH_{SO}

Con A-Sepharose Affinity Chromatography of EH_{SO}

SDS-polyacrylamide gel analysis of the unbound and bound eluates acquired from samples passed through Con A columns revealed that EH_{SO} is not glycosylated. EH_{SO} was eluted in the unbound fraction indicating that it did not bind to the Con-A-sepharose column which is specific for glycoproteins (see Fig. 8).

Gel Immunidiffusion of EH_{SO}

Antibody raised to homogeneous fish liver microsomal epoxide hydrolase was used to identify EH_{SO} activity in the crude microsomal fractions and the purified enzyme. Single immunoprecipitin lines were observed in all fractions that contained microsomal EH_{SO} activity (see Fig. 9a). To ascertain that the antiserum was specific for the microsomal form of enzyme isolated, a crude cytosolic preparation was also used in the analysis. Figure 9b shows no immunoprecipitin line was observed in the cytosolic fraction.

Titration of mRNA in an In Vitro Translation System Using Rabbit Reticulocyte Lysate

Titration of free and membrane-bound polysome preparations and total RNA was initially done to determine the amount of the RNA in the system which is most efficient in the translation of proteins. The incorporation of [^{35}S-] methionine into proteins was monitored for 5 minutes in the Beckman LS 7500 scintillation counter spectrophotometer. Figure 10 summarizes the results obtained. The titration of FP show

that 3 μl aliquot of FP preparation, which corresponds to 15.65 μg/ml of mRNA, is most efficient in the synthesis of proteins in the system used and that RER-MBP and total RNA exhibit the highest efficiency at 4 μl aliquots, which corresponds to 4.1 μg/ml of mRNA and 38.50 μg/ml of mRNA, respectively.

In Vitro Synthesis of EH_{so}

In vitro translation of proteins in a cell-free rabbit reticulocyte lysate system was achieved by utilizing total RNA, or free or membrane-bound polysomal preparations isolated from styrene oxide treated rat liver. Table 3 shows the amount of incorporation of [^{35}S]-methionine into proteins in a 25 μl translation mixture. Total [^{35}S]-methionine incorporation in 25 μl samples was 1.53 x 10^5 counts/min in FP and 1.16 x 10^5 counts/min in extracted total RNA. The cotranslational addition of exogenous membrane (DPM) appeared to cause inhibition in the translational system incorporation of [^{35}S]-methionine into proteins. The addition of 5 μl of DPM to the 25 μl samples produced 1.66 x 10^5 counts/min in FP and 1.05 x 10^5 counts/min in extracted total RNA.

Immunoprecipitation of EH_{so}

SDS-polyacrylamide gel electrophoresis and autoradiography reveals that epoxide hydrolase was immunoprecipitated from translation mixtures that were programmed with total RNA, free or membrane bound polysome preparations. (Table 3 and Figs. 11 and 12). The immunoprecipitable epoxide hydrolase has a molecular weight of 56,000 daltons.

Co-translational Processing and Post-translational Proteolysis of the In Vitro Synthesized EH_{SO}

The addition of stripped dog pancreatic microsomal membrane (DPM) to *in vitro* translation mixtures programmed with free polysomes was done to determine if the immunoprecipitable EH_{SO} was a precursor of the MBP form (Fig. 11, lane 2). The results showed no difference in the FP preparation plus DPM immunoprecipitable EH_{SO}. The protein immunoprecipitated has a molecular weight of 56,000 daltons. The total RNA preparation was also used as a probe with the addition of DPM in hopes of finding a difference once the protein was processed (inserted into the membrane). The results also yield a 56000 daltons protein (Fig. 12 lane 2).

To test whether the DPM was functional, an IVT was done which contained MBP, FP-DPM or FP+DPM. The translational products were digested posttranslationally and analyzed. Results indicated that the DPM was in fact functional because the IVT products of the FP+DPM preparation was protected from proteolysis whereby the FP-DPM preparation was digested (Fig. 13). Immunoprecipitable EH_{SO} from MBP, FP+DPM or FP-DPM translation mixtures were also proteolytically digested post-translationally and the results also show that the "FP+DPM" was protected (Fig. 14).

Thus, these results suggest that the FP preparation contain detached RER polysomes.

Fig. 8. Protein profiles of the eluates from Con-A Sepharose affinity chromatography of EH_{SO}.

Gel 1 - shows the profile of fraction applied to column.

Lane 1 - molecular weight standards;

Lane 2 - protein profile of sample;

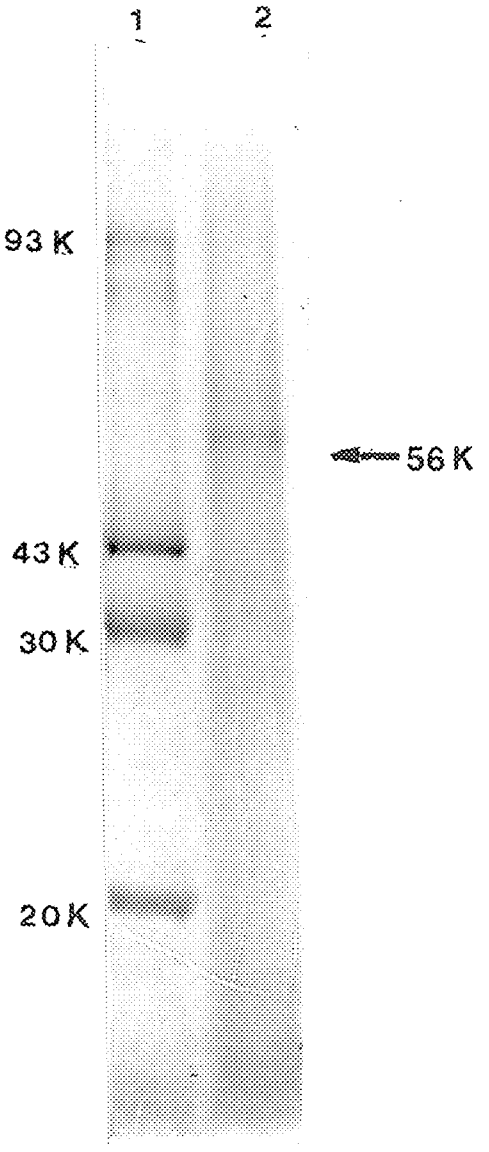
(DEAE eluate) before application to Con - A column.

Gel 2 - shows the profile of fractions eluted from Con A-column:

Lane 1 - molecular weight standards;

Lane 2 - unbound fraction eluate;

Lane 3 - bound fraction eluate.


1
2
93 K
56 K
43 K
30 K
20 K

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Co-translational Processing and Post-translational Proteolysis of the In Vitro Synthesized EH_{SO}

The addition of stripped dog pancreatic microsomal membrane (DPM) to *in vitro* translation mixtures programmed with free polysomes was done to determine if the immunoprecipitable EH_{SO} was a precursor of the MBP form (Fig. 11, lane 2). The results showed no difference in the FP preparation plus DPM immunoprecipitable EH_{SO}. The protein immunoprecipitated has a molecular weight of 56,000 daltons. The total RNA preparation was also used as a probe with the addition of DPM in hopes of finding a difference once the protein was processed (inserted into the membrane). The results also yield a 56000 daltons protein (Fig. 12 lane 2).

To test whether the DPM was functional, an IVT was done which contained MBP, FP-DPM or FP+DPM. The translational products were digested posttranslationally and analyzed. Results indicated that the DPM was in fact functional because the IVT products of the FP+DPM preparation was protected from proteolysis whereby the FP-DPM preparation was digested (Fig. 13). Immunoprecipitable EH_{SO} from MBP, FP+DPM or FP-DPM translation mixtures were also proteolytically digested post-translationally and the results also show that the "FP+DPM" was protected (Fig. 14).

Thus, these results suggest that the FP preparation contain detached RER polysomes.

Fig. 8. Protein profiles of the eluates from Con-A Sepharose affinity chromatography of EH_{SO}.

Gel 1 - shows the profile of fraction applied to column.

Lane 1 - molecular weight standards;

Lane 2 - protein profile of sample;

(DEAE eluate) before application to Con - A column.

Gel 2 - shows the profile of fractions eluted from Con A-column:

Lane 1 - molecular weight standards;

Lane 2 - unbound fraction eluate;

Lane 3 - bound fraction eluate.

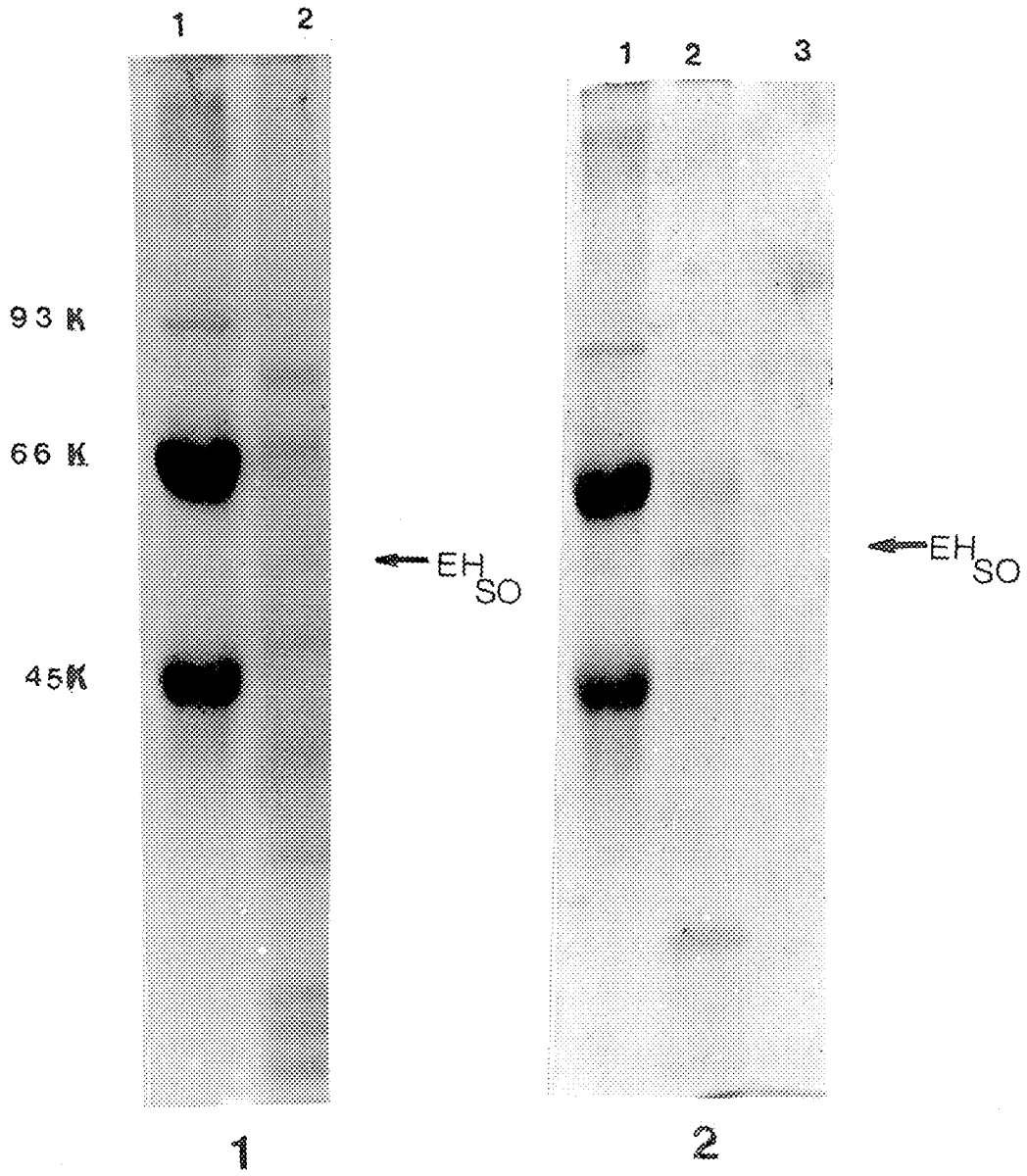
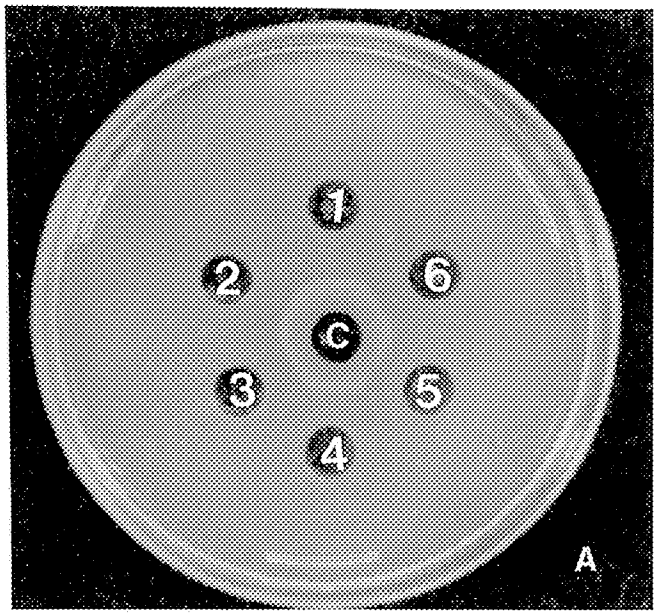

1
2
93 K
66 K
45K
EH SO
1
1
2
3
EH SO
2

Fig. 9. Ouchterlony immunodiffusion analysis of epoxide hydrolase

Ouchterlony plates were prepared in 2% agarose gels. Center wells contain 20 μg of antiserum to EH. Plate a: Well 1- Solubilized microsomes (50 μg); well 2-SM (50 ug); Well 3 - RM (50 μg); Well 4-PMS (50 μg); Well 5 - CM eluted EH_{so}; (10 μg) Well 6 - DEAE eluates that contain EH_{so}.

Plate b: Well 1 - borate - buffered saline; well 2 - cytosol fraction; Well 3 - empty; Well 4 - EH_{so} (10 μg); Well 5 - crude microsomes (20 μg); Well 6 - Lubrol-solubilized microsomes.


1
2
6
C
3
5
4
A

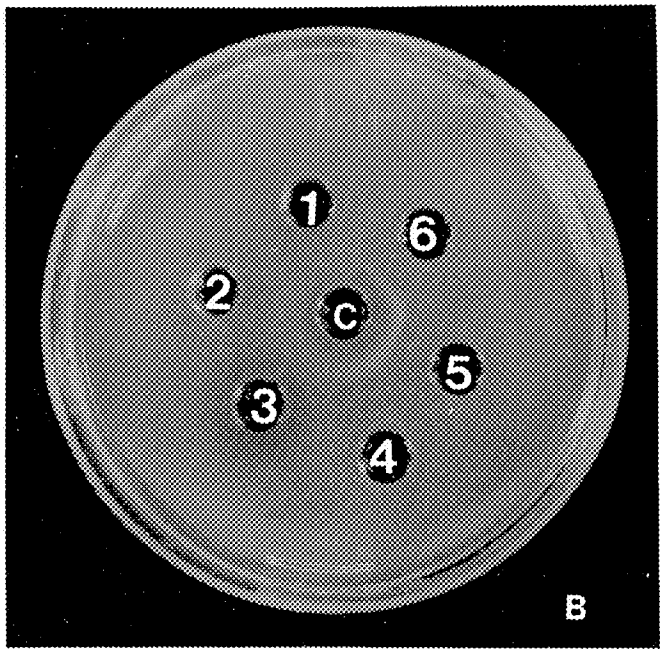

1
6
2
C
5
3
4
B

Fig. 10. A graph showing the titration of m RNA in an IVT system using FP, MBP and extracted RNA (total).

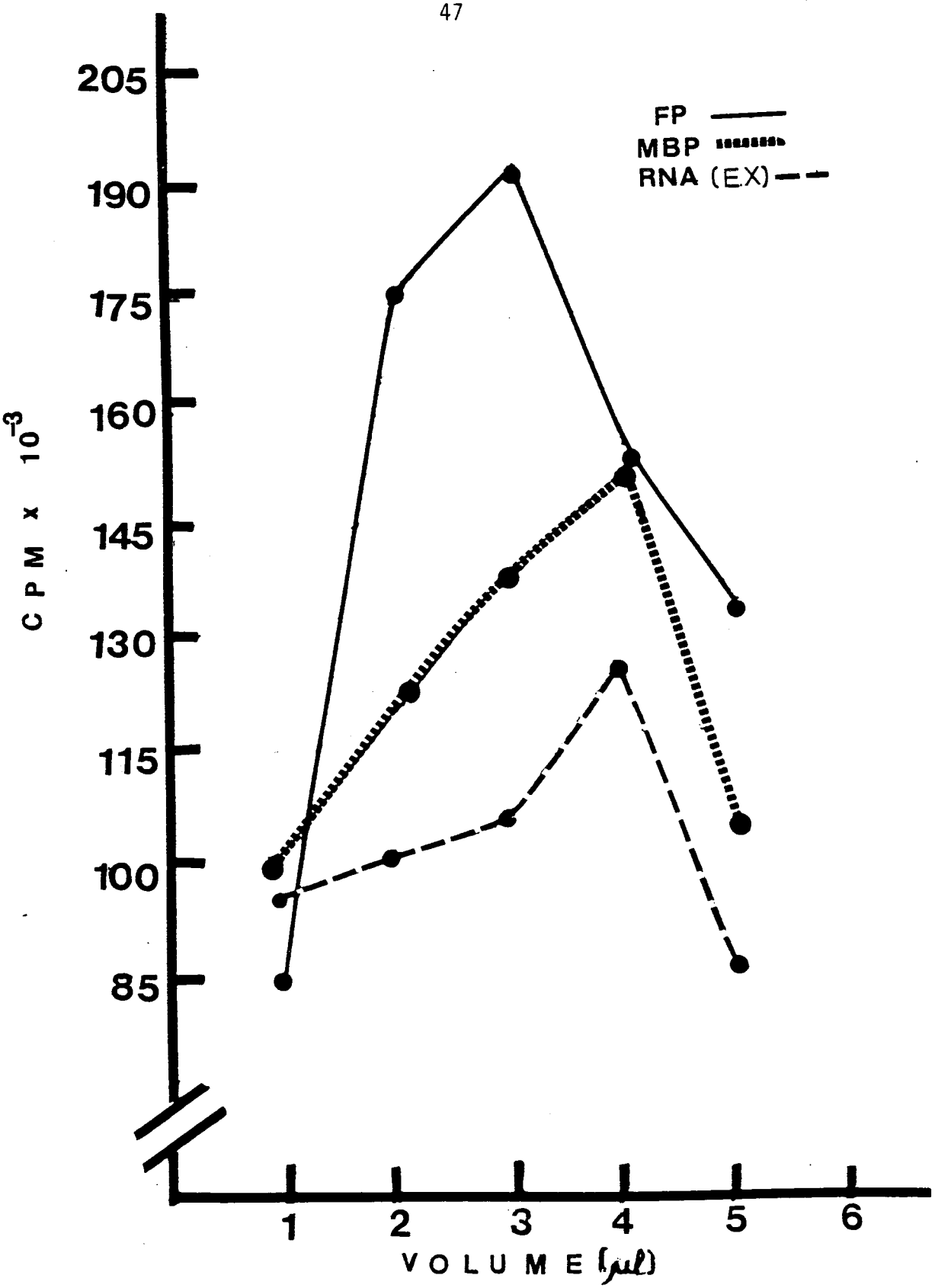

205
190
175
160
145
130
115
100
85
CPM x 10^{-3}
FP
MBP
RNA (EX)
1
2
3
4
5
6
VOLUME (μl)

Table 3. Incorporation of [^{35}S]-methionine in an *in vitro* translation system using a rabbit reticulocyte lysate system.

mRNA Source	Total [^{35}S]-methionine Incorporation Into Proteins (CPM)	Immuno-Precipitable EH_{SO} (CPM)
Endogeneous Lysate	297867	---
RER-MBP	152970	1530
FP	192626	2431
FP+DPM	165950	1634
RNA (Total)	115630	1057
RNA (Total) + DPM	105367	1296
Digested Lysate	87521	---

Fig. 11. Autoradiogram of SDS - gel showing immunoprecipitable EH_{SO} from IVT products.

The system was programmed with RNA derived from RER-MBP or FP: Lane 1 - RER-MBP EH_{SO}; Lane 2 - FP + DPM EH_{SO}; Lane 3 - FP - DPM EH_{SO}.

Arrow indicates the immunoprecipitated EH_{SO}.

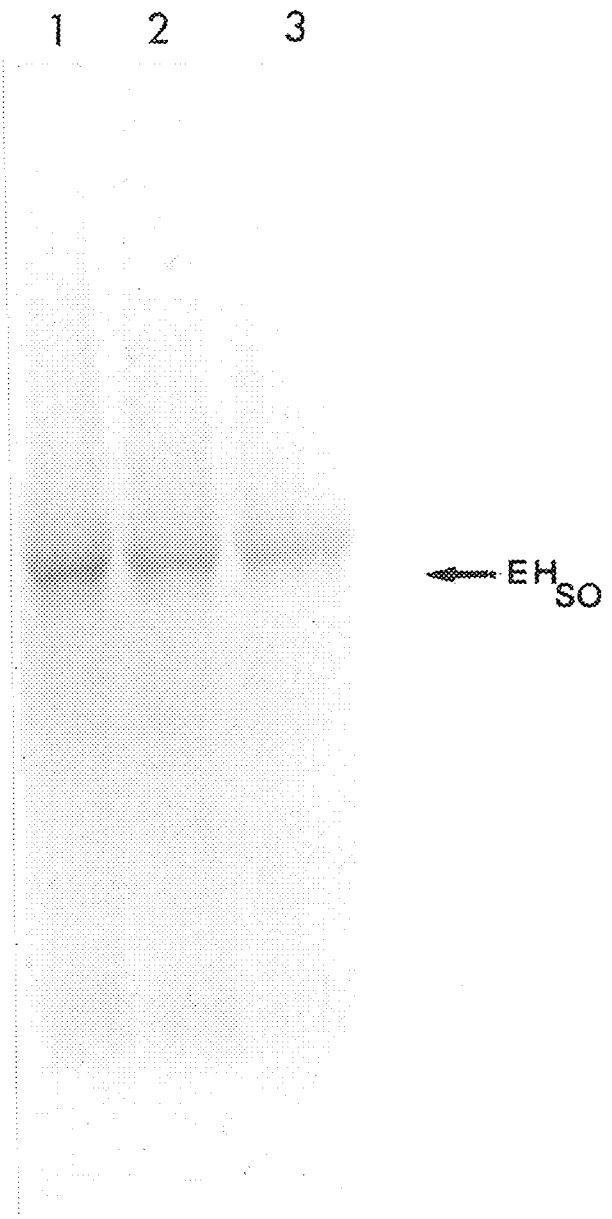

1
2
3
EH$_{SO}$

Fig. 12. Autoradiogram of SDS-gel showing immunoprecipitable EH_{SO} from IVT products using extracted total RNA.

Lane 1 represents total RNA EH_{SO}; Lane 2 represents total RNA EH_{SO} in the presence of DPM.

Arrow indicates the immunoprecipitated EH_{SO}.

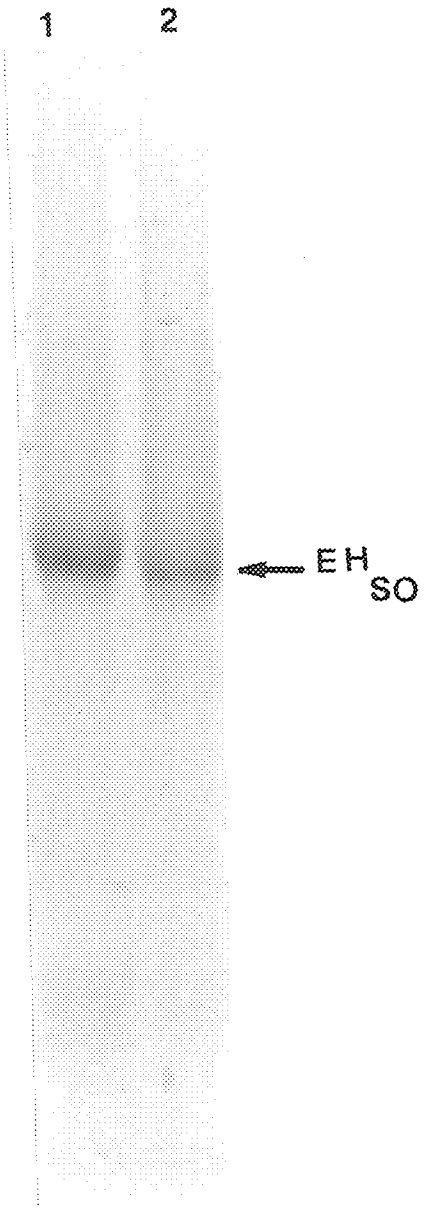

1
2
EH
SO

Fig. 13. Autoradiogram of SDS-gel of the IVT products following co-translational and post-translational proteolysis.

Lane 1 - Molecular weight standards;

Lane 2 - RM-MBP translated products;

Lane 3 - FP - DPM translated products.

Lane 4 - FP + DPM translated products

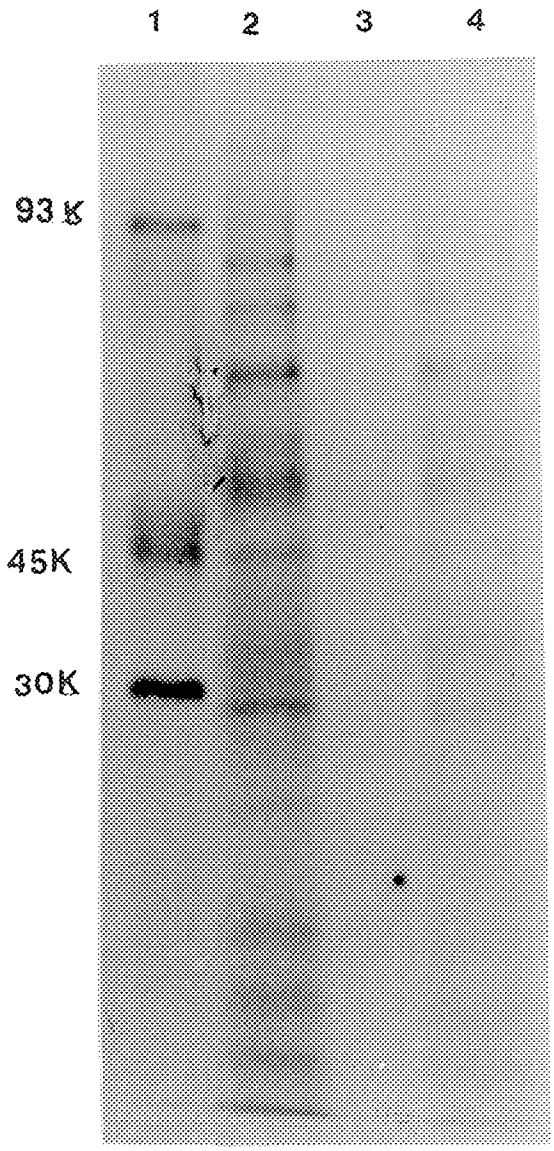

1
2
3
4
93 K
45K
30K

Fig. 14. Autoradiogram of SDS-gel of immunoprecipitable EH_{SO} following cotranslational processing and posttranslational proteolysis.

Lane 1 - FP - DPM; Lane 2 - RER-MBP;

Lane 3 - FP + DPM.

Arrow indicates the immunopreicipitated EH_{SO}.


1
2
3
EH
SO

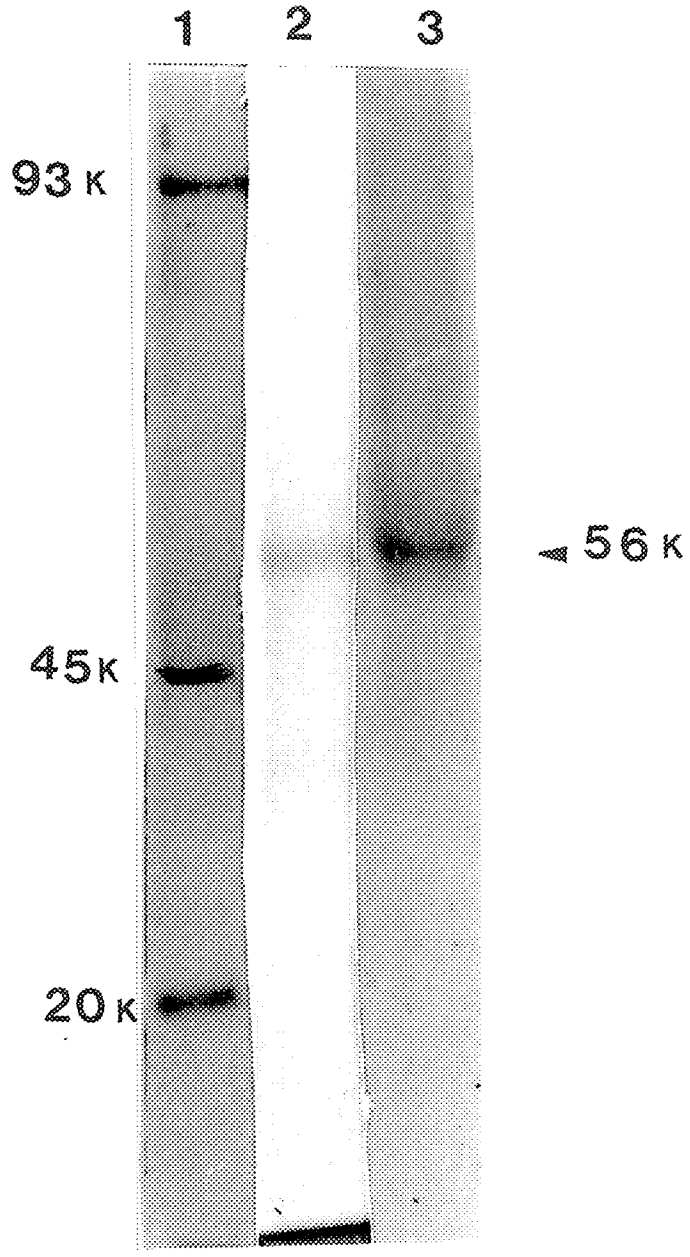
Comparison of the In Vivo EH_{so} with the In Vitro EH_{so}

The partially purified EH_{so} was eluted from CM cellulose column electrophoresed on the same gel with the immunoprecipitable EH_{so} from *in vitro* translation products. Results indicated the authentic (*in vivo*) form comigrates with the *in vitro* form (Fig. 15). *In vivo* and *in vitro* EH_{so} have a molecular weight of 56000 daltons.

Fig. 15. SDS - PAGE comparison of the *in vivo* and *in vitro* EH_{SO}

Lane 1 - Radioactive molecular weight markers; Lane 2 - SDS-PAGE of pure EH_{SO}; Lane 3 - autoradiogram of *in vitro* synthesized EH_{SO}. Proteins comigrate in SDS-PAGE

Arrow indicates EH_{SO}.


1
2
3
93 K
45K
20 K
56 K

CHAPTER V

DISCUSSION

Epoxide hydrolase is an enzyme which plays a very important role in the metabolism of drugs, mutagens and/or carcinogens in a number of naturally occurring, as well as synthetic compounds (Oesch et al, 1971; Thomas et al, 1981; Seidegard and DePierre, 1983; Kuhlman et al, 1981). Several investigators have demonstrated that phenobarbital (Gonzales and Kasper 1980; Pickett and Lu, 1981) and 3-methylcholanthrene (Okada et al, 1982; Guengerich et al, 1979) cause induction of mRNA programmed for the synthesis of epoxide hydrolase. The induction of synthesis of epoxide hydrolase has played a major role in understanding the complex cascade of reactions involved in the metabolism of xenobiotics and the functional site of the enzyme. Simultaneously, these investigators have definitively shown that PB and 3-MC induced epoxide hydrolase is synthesized on membrane bound polysomes of the rough endoplasmic reticulum. Both of these chemicals under go the same fate in the cell. Phenobarbital and 3-methylcholanthrene are procarcinogens. They are first activated by cytochrome P-450 to their reactive products prior to causing induced synthesis of epoxide hydrolase. However, many chemicals are direct acting carcinogens (Hemminki, 1983) and exist as highly reactive products in the cell. Those highly reactive compounds also cause changes in the levels of enzymes responsible for their metabolism. The induced

synthesis of epoxide hydrolase after exposure of laboratory animals to direct acting carcinogens have not yet been reported.

The investigation conducted in this laboratory has focused on understanding the role of styrene oxide, a direct acting carcinogen, in the induction of epoxide hydrolase. Styrene oxide induced the synthesis of a form of epoxide hydrolase (EH_{SO}) in rat liver microsomes *in vivo* and also *in vitro* in a rabbit reticulocyte lysate cell-free system using RER-MBP, FP and total RNA from SO exposed liver. Subcellular fractionation of SO treated livers on a discontinuous sucrose gradient provided the source for the translational machinery (ribosomes) needed in the *in vitro* synthesis of EH_{SO}. *In vitro* translations using polysomes attached to membranes (RER-MBP) have been shown to synthesize EH_{SO}. These results initially suggested the existence of two different forms of the enzyme of the same molecular weight. Both membrane-bound polysomes and free polysomes directed the synthesis of a 56K polypeptide. Stripped DPM was cotranslationally added to the FP and resulted in the processing of two forms of the polypeptide, with the same molecular weight. That is, the RER-MBP, FP - DPM and FP +DPM all synthesized a 56K polypeptide. However, further results using free polysomes in the presence of the exogenous ribosomally stripped dog pancreatic microsomal membrane (DPM) synthesized a 56K protein which was not susceptible to proteolytic digestion (posttranslationally). Free polysomes devoid of microsomal membrane (DPM) were subjected to posttranslational proteolysis and

was not protected. This set of data provide conclusive evidence that the free polysomal preparations do not consist entirely of free polysomes and strongly suggest that the FP contain detached RER-MBP, which are capable of inserting into membranes. Detachment of ribosomes from the RER is one of the most conspicuous ultrastructural alterations observed during hepatocarcinogenesis induced by a number of chemical carcinogens (Svoboda and Higginson 1968; Fox et al, 1984). This situation may explain why styrene oxide induced epoxide hydrolase (EH_{SO}) appears to be synthesized on FP but in fact, this may not be the case, since SO may cause detachment of ribosomes from the RER.

The preceeding observations demonstrate that perturbed and detached membrane-bound polysomes from the livers of SO treated rats are still capable of synthesizing epoxide hydrolase in a cell-free systeme. These findings are in agreement with studies from other laboratories (Okada et al 1982; Gonzales and Kasper, 1984; Pickett and Lu, 1981).

Partial purification of SO induced epoxide hydrolase has been achieved by detergent solubilization and ion exchange chromatography. The protein is homogeneous as judged by SDS polyacrylamide gel electrophoresis and has a molecular weight of 56,000 daltons. Isolation of EH_{SO} from endomembranes begs the question as to what is involved in its processing. For example, is it glycosylated? To answer this question, the ability of EH_{SO} to bind specifically to a Con -A sepharose affinity column was tested. Con A is a plant lectin which binds mannose residues

present in oligosaccharides. Binding is specifically prevented by competition with a-methyl mannoside. The results obtained by the Con A affinity column binding studies showed that EH_{SO} is not a glycoprotein since it was eluted from the column in the nonbinding fraction.

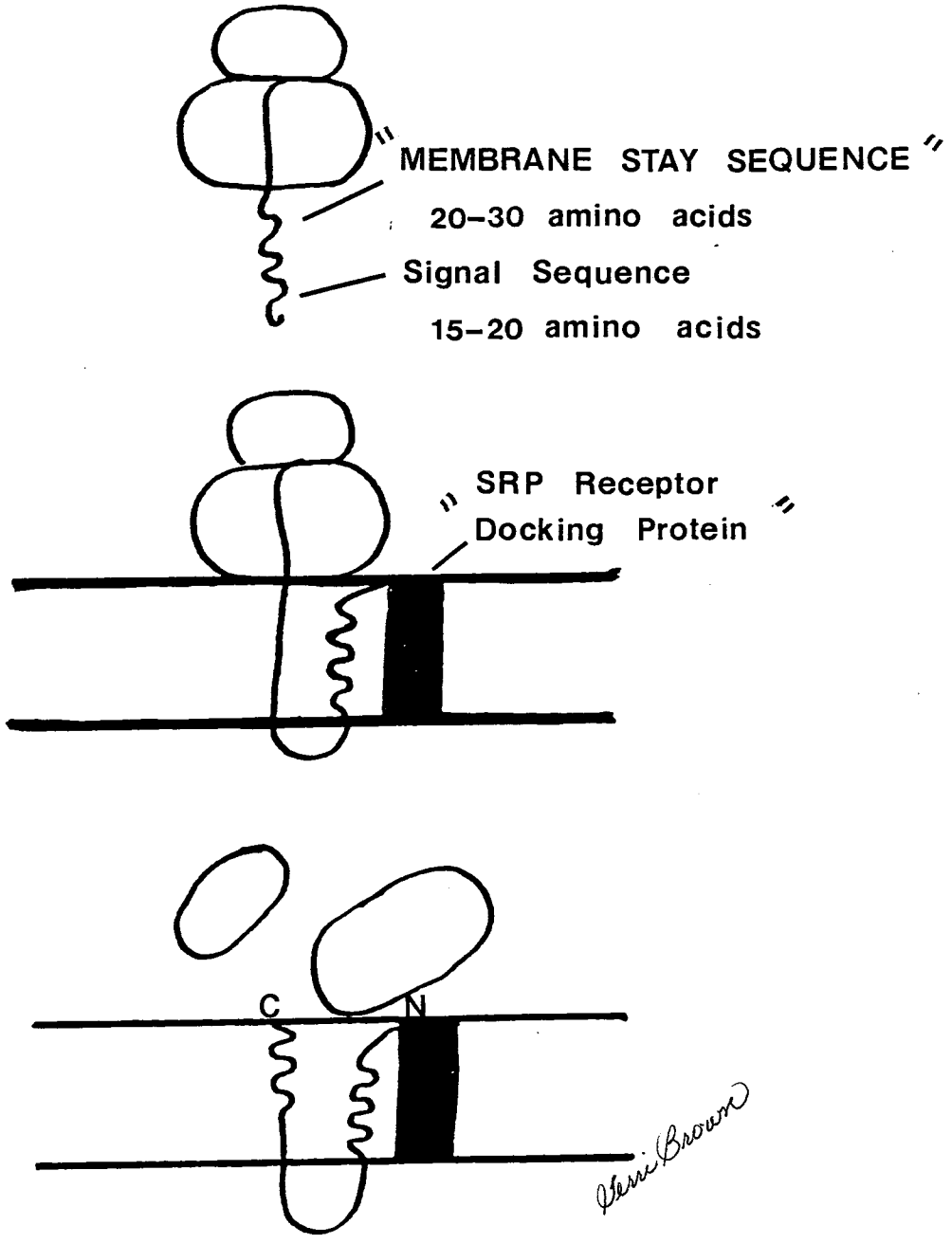
The ability of the enzyme to hydrate two distinct substrates suggests that it is a different form of the enzyme not previously reported. However, this must be validated by its amino acid sequence analysis, which is proposed as a project to be undertaken in this laboratory. Immunologically, this enzyme has been shown to be cross reactive with rabbit anti-fish-epoxide hydrolase (isolated in our laboratory) (Jideama and Browne, 1985).

The enzyme synthesized in vitro comigrated on an SDS polyacrylamide gel with the purified native polypeptide (in vivo). Comigration of the in vitro product with the authentic (in vivo) form suggest that the enzyme has a noncleavable signal sequence. These data are also in agreement with previous investigators (Ohlsson et al, 1981; Okada et al 1982). In addition to the presence of a noncleavable sequence, the post translational proteolysis of this form of epoxide hydrolase (EH_{SO}) suggest that the enzyme is deeply embedded in the membrane with no portion of it cytoplasmically exposed.

Sabatini et al (1982) suggested that signals for cotranslational insertion of membrane polypeptides need not always be transient and therefore, may remain in the mature protein. The data in our studies suggest that the amino terminal sequence of the primary translation product of EH_{SO} is not removed. The EH_{SO} synthesized in vitro using total RNA in the absence of added microsomal membranes was found to correspond to that of the mature product. This suggests, since EH is a putative endomembrane protein, corroborative support for the hypothesis advanced by Sabatini et al (1982).

Heineman and Ozols (1984) have shown that the amino terminal segments of epoxide hydrolase is rich in hydrophobic amino acids and resembles the signal sequences present in pre-secretory proteins. It is suggested that these sequences function as signals for cotranslational insertion. Sabatini et al (1982) reported that if the amino terminal is the putative cotranslational "insertion signal" of epoxide hydrolase then one might have expected, by analogy with secretory proteins, that most of the molecule would pass through the membrane leaving only a segment of the C-terminal portion attached to it on the cytoplasmic side by the permanent insertion segments. A priori, it is important to point out that a portion of the epoxide hydrolase molecule is known to be exposed on the cytoplasmic side (Seidegard et al 1983) of the microsomal membrane, suggesting that this polypeptide also contains a "halt transfer" signal that interrupts cotranslational transfer through the membrane leaving a portion of the molecule on the cytoplasmic surface.

Unlike, the models proposed by Sabatini et al, (1982) we have proposed a hypothetical model (Fig. 16) to describe the role of the amino terminal cotranslational insertion signal of EH. The model shows the insertion of the integral membrane protein EH_{SO} into the endoplasmic reticulum. The model suggests that EH_{SO} has putative "membrane stay sequences." We are suggesting that the presence of these sequences prevents the vectoring of the nascent polypeptide through the endomembrane system. We believe that the protein is processed to its authentic form as it is inserted into the endoplasmic reticulum. The model also suggest that the protein is not exposed at the cytoplasmic surface of the membrane and it is deeply embedded in the membrane.


" MEMBRANE STAY SEQUENCE "
20–30 amino acids
Signal Sequence
15–20 amino acids
" SRP Receptor
Docking Protein "
C
N
Jessi Brown

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summarily, the data reported in this study show that:

1. Styrene oxide is an inducer of microsomal epoxide hydrolase in rat liver;
2. Partial purification and enzymatic activity suggest that SO induces a form of epoxide hydrolase in rat liver microsomes;
   This form of microsomal epoxide hydrolase is inducible with 500 mg of styrene oxide in adult male Long-Evans rats within 48 hr post-intraperitoneal injection;
   This form of epoxide hydrolase is electrophoretically resolvable on a 7.5% SDS-polyacrylamide gel and has a relative molecular weight of 56,200;
3. *In vivo* and *in vitro* synthesis of EH_{SO} suggest that it is synthesized on membrane bound polysomes and has a noncleavable signal sequence;
4. Concanavalin A binding studies suggest that EH_{SO} is not a glycoprotein;
5. This form of epoxide hydrolase is immunologically similar to fish liver microsomal epoxide hydrolase (49,000 dalton form) identified in our laboratory.

We have determined from the experimentally derived data delineated above that the sites of biosynthesis of styrene oxide-induced epoxide hydrolase are the ribosomes attached to the "rough" endoplasmic reticuli. Moreover, the putative mode of insertion appears to be by the presence of a noncleavable amino-acid sequence. This is inferred from the experimental results showing no molecular weight differences in the "pre-protein" synthesized in vitro, in the absence of dog pancreas microsomal or in its presence. That is to say, the "preprotein" molecular weight moeity is the same as that of the authentic form. The pertubation and detachment of membrane-bound polysomes by exposure to styrene oxide, a known carcinogen, though not surprising, was unexpected and afforded some pertinent resulting data. Ribosomes which appear to be "free" and considered to routinely synthesize cytoplasmic proteins are not always functionally the same. The presence or absence of a putative messenger RNA containing a "signal sequence" for directing translation of a polypeptide in association with a microsomal membrane, if present, appears to be the more definitive vector.

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Figure 16 - Hypothetical model for EH_{SO} cotranslational insertion into the endoplasmic reticulum membrane. Protein has putative "membrane stay sequence."